INFLUENZA VIRUS OF THE DEVELOPING EGG

5. DIFFERENTIATION OF TWO ANTIGENIC TYPES OF HUMAN INFLUENZA VIRUS

by

F. M. BURNET[1]

(From the Walter and Eliza Hall Institute, Melbourne).

(Submitted for publication 13th September, 1937.)

Until recently it was currently stated that all strains of human influenza virus were antigenically identical, but could be sharply differentiated from the swine type virus, which possessed only minor antigenic elements in common. Actually the evidence for this statement was based almost entirely on the use of the immune horse serum I.H.2 of Laidlaw, Smith, Andrewes, and Dunkin (1935). As will be shown, this serum is far less specific in its neutralizing power than are convalescent sera from susceptible animals such as ferrets. A hint that there were specific differences between strains was obtained when Dr. C. H. Andrewes tested serum from a ferret convalescent from the "Melbourne" strain against strain "W.S." He found that although it neutralized the virus its titre was definitely below that of I.H.2. In Melbourne, on the other hand, we had found that the same ferret serum neutralized the homologous virus to a higher titre than I.H.2 (Burnet, 1936a). Owing to the possibility that both samples of sera which travelled across the tropics might have suffered serious deterioration of titre, the difference was not further studied until the work of Magill and Francis (1936) appeared. They found on immunizing rabbits with the human strains "Philadelphia" and "P.R.8" (from Porto Rico) and Shope's swine influenza virus, that the sera showed relatively specific neutralizing powers and that with such sera the strain "P.R.8." showed a closer relationship to the swine strain than to the other human strain.

Similar studies of the antigenic relationships amongst influenza virus strains are being carried on in other laboratories, and in this paper the main stress will be laid on the results obtained by the quantitative egg membrane technique, which is not in use overseas, so as to avoid as far as possible overlapping English and American work.

The following strains of human influenza virus have been studied: "W.S.", England 1933, "B.H.", England 1935, "Leningrad", 1936 (Smorodintseff *et al.*,

[1] Work supported by a grant for research on virus diseases from the Rockefeller Foundation and the Commonwealth Government Department of Health.

1936), "Moscow", 1936 (Silber), "Philadelphia", 1934, "P.R.8", Porto Rico, West Indies, 1934, and "Melbourne", 1935. The first four strains were obtained through the kindness of Dr. C. H. Andrewes, and the two American strains from Dr. T. Francis, Jnr. All were received in the form of dried mouse lung, and all proved typically virulent for mice and ferrets. A strain of swine influenza virus was also received from Dr. Andrewes.

The development of an egg pathogenic strain of "Melbourne" virus and the result of immunological work with this technique have already been described (Burnet, 1936 a, b, c). In the course of the present work, two other egg pathogenic strains have been developed from swine virus and "W.S." The "W.S" strain became rapidly adapted to the chorioallantois, producing good focal lesions by the fifteenth passage and killing the embryo within three days by the twentieth passage. The swine influenza strain has so far proved less readily adaptable. At present it has had over fifty passages on the chorioallantois, but is much less active than "W.S." at twenty passages. The foci are small and are not clearly visible till three days after inoculation. Their numbers are more variable than those of well adapted strains, and satellite formation is often sufficient to render membranes useless. The embryo is never killed. Details of the process of adaptation of these strains to the egg will be published elsewhere.

The methods used in titration of virus and antisera on the chorioallantois have been fully described for the Melbourne strain (Burnet, 1936 b, c) and were used unchanged for the other two strains, except that eggs used in swine influenza experiments were opened three days after inoculation instead of two. The results with "W.S." are as reliable as those with "Melbourne", but a wider margin of error must be allowed with "Swine".

Cross Neutralization Tests with Convalescent Ferret Sera.

In Table 1 are combined a series of titrations of the antibody content of convalescent ferret sera against the three strains "Melbourne", "W.S.", and "Swine". The ferrets were bled three to four weeks after intranasal inoculation with mouse virus of the type shown, with the exception of Ferret 51, which was infected by contact with another ferret given the "Melbourne" strain. All ferrets gave a febrile reaction of the usual diphasic form.

A complete series of egg membrane titrations is given, but owing to limitations in the supply of mice available, only a few of the possible serum-virus combinations have been tested by the commonly used method of intranasal inoculation in mice. It will be seen that, on the whole, results of mouse titrations run closely parallel (as regards specificity at least) to the egg membrane titrations. The quantitative differences shown between the two methods will be discussed elsewhere.

The results in Table 1 show clearly that, as judged by the reactivity of convalescent ferret sera taken soon after infection, there are three sharply defined groups of influenza virus strains. The first contains the human strains "Mel-

bourne", "P.R.8", and "Philadelphia", the second the two English and the two U.S.S.R. strains, and the third swine influenza virus. There are indications of minor differences within each group.

TABLE 1.

Immunological Reactions of Immune (Convalescent) Ferret Sera.

Ferret No.	Strain used for infection of ferret.	Antibody titre when tested against: Melbourne. Egg.	Melbourne. Mouse.	W.S. Egg.	W.S. Mouse.	Swine. Egg.	Swine. Mouse.
F.51	Melbourne	800	2,000		<10	0·08	<1
F.67	"	330	1,250	0·8	(<50)	0·1	
F.57	P.R.8	210	1,200			1·4	<10
F.61	"	100		0·5		0·2	
F.58	Philadelphia	160				0·11	<10
F.62	"	800		0·25		0·38	
F.63	W.S.	0·9	<50	150	350(1,000)	0·42	
F.64	B.H.	0·6		57		0·5	
F.65	Leningrad	0·34	<50	34	(300)	0·3	
F.66	Moscow	0·47		83		0·3	
F.55	Swine	0·07		0·1		400	500
F.56	"	0·12	1·0	0·08		250	1,000
I.H.2	Horse serum W.S.	13	200	36	70	50	25

Egg titration results are shown as the reciprocal of the percentage reduction in number of foci produced by mixing undiluted serum with an equal volume of virus. The relation C (concentration of antibody) × P (proportion of survivors) = constant (see Burnet, Keogh and Lush, 1937) is assumed to hold throughout. As an example, if a serum diluted 1: 100 mixed with stock virus gives 0·1 p.c. of the original count of foci, undiluted serum should reduce the count to 0·001 p.c. and the value given in the table would be 1,000.

The results of mouse titrations are expressed as ten times the dilution of serum which, mixed with undiluted stock virus, gives lung lesions involving 40–50 p.c. of the lung surface when mice are killed at six days. Values are obtained by interpolation from the degrees of consolidation observed with a series of five-fold serum dilutions, three mice being used for each mixture. Stock virus is an unfiltered supernatant fluid prepared from one consolidated lung in 5 c.c. broth, except for the bracketted results, for which an Elford membrane filtrate was used.

Included in the table are results obtained in the same experiments with a sample of the immune horse serum of Laidlaw *et al.* (1935), I.H.2. This is now more than two years old, and its activity may have fallen somewhat. It will be seen that this serum shows only relatively slight differences between the three types. The lack of specificity of this serum is probably due to the prolonged hyper-immunization of the horse supplying it.

Differences between Strains as Shown by Active Immunity in Mice and Ferrets.

It has been shown previously that mice could be immunized by the introduction intranasally of almost non-pathogenic egg-adapted virus ("Melbourne" strain) against highly active mouse-adapted virus of the same type (Burnet, 1937). When these experiments were extended to other strains (Table 2) it was found that protection was equally evident against the strain "Philadelphia" and to a very slight extent against "P.R.8". The strains "W.S." and "Leningrad"

however caused fatal infections in all the immunized mice tested. In all these experiments the test for immunity was the very severe one of intranasal inoculation of undiluted stock virus equivalent in each case to more than 1,000 lethal doses, so that only high grades of immunity could be detected. Taking into account the distinct prolongation of survival time shown with some of the strains which proved fatal to the immunized mice, it seems probable that a considerable degree of heterologous immunity would be demonstrated with smaller test doses than those used.

TABLE 2.

Action of Different Strains of Human Influenza Virus on Mice Immunized with Melbourne Egg Virus.

	Experiment 1.						Experiment 2.					
Test virus.	Immunized mice.			Controls.			Immunized mice.			Controls.		
Melbourne	S	S	S	3	3	4	S	S	S	2	2	3
Philadelphia	S	S	S	3	4		S	S	S	4	4	4
P.R.8	5	5	5	4	4		3	6	S	3	3	3
Swine	5	5	5	4	5							
W.S.							3	3	3	3	4	
Leningrad							4	5	6	3	3	3

Figures indicate days to death. S = Survival for 7 days.

Active immunity in ferrets has not yet been adequately tested, but the following experiment, carried out primarily for another purpose, indicates that there is little active immunity against the heterologous type of virus. Two ferrets, F.59 and F.60, were given "Melbourne" egg virus intranasally. Neither showed any temperature reaction. A fortnight later they were tested along with two control ferrets with "P.R.8" (F.59) and "Philadelphia" (F.60) strains. Neither F.59 nor F.60 reacted, but the control ferrets showed typical temperature curves with maxima of 106·8° F. (P.R.8") and 105·4° F. ("Philadelphia"). During the next three months they were given several subcutaneous inoculations of one or other strain of the American-Australian group. After a blood sample had been taken and titrated, both ferrets were given an intranasal inoculation of strain "W.S." Both gave a well-marked febrile reaction of diphasic type with maxima of 105·3° F. and 105·5° F., but symptoms were slight.

Antibody Absorption Experiments.

It is possible to absorb out virus-inactivating antibody by treating diluted antiserum with virus-containing tissue. Work in progress has shown that the antibody responsible for the reduction in activity shown with egg membrane titrations is much more readily removed than that manifested in the results of titrations in mice. It was of interest to compare the results of absorption of a ferret serum containing "Melbourne" type antibody, with each of the available strains in the form of consolidated mouse lung.

The technique was as follows: 2 c.c. of serum F.67 ("Melbourne") diluted 1:40 were added to a paste of two finely-ground consolidated lungs: the emulsion was shaken and left overnight in the refrigerator. Next day the emulsion was centrifuged and the supernatant fluid heated to 56° C. for 30 minutes to destroy any active virus. This caused a copious precipitation and a second centrifugation was carried out, the final supernatant fluid representing the absorbed serum. Numerous control tests have shown that there is no loss of antibody activity by nonspecific processes in such manipulations. Actually if a control serum diluted to the same extent is heated in the same way it suffers more damage than sera mixed with a dense tissue emulsion, and if there is no specific absorption the "absorbed" serum appears stronger than the control. The absorbed and control sera are titrated on the egg membrane according to the usual technique, and the percentage reduction in antibody can be easily calculated, e.g. if control serum reduces the focal count to 0·24 p.c., and serum absorbed with "Melbourne" virus to 6·0 p.c., the latter serum has $\frac{1}{25}$th the activity of the control, i.e. there has been a reduction in antibody activity of 96 p.c.

The absorption results showed the same well-marked differences amongst the virus strains as were obtained by other methods. The strains "Swine", "W.S.", "Moscow", and "Leningrad" showed no detectable absorption under these experimental conditions, while with the three strains of the "Melbourne" group the following percentage reductions in antibody activity were obtained: "Melbourne", 96 p.c., "Philadelphia" 93·3 p.c., and "P.R.8" 84 p.c.

DISCUSSION.

We may summarize the evidence as showing that there is a certain basal antigenic resemblance between all influenza virus strains, allowing some degree of cross reaction, but that active and passive immunity experiments agree in dividing the strains into three distinct groups. These may be termed the Old World human type, exemplified by "W.S.", the New World human type, exemplified by "Melbourne", and the swine type, Shope's strain "15" being that in current use in most laboratories. Within each of the two human types there may be minor antigenic differences. Magill and Francis (1936) found, for instance, that well-marked antigenic differences between the strains "P.R.8" and "Philadelphia" could be detected by neutralization tests made with immune rabbit sera. Such tests also showed an unexpectedly close antigenic relationship between "P.R.8" and swine strains. In our experiments "Philadelphia" and "Melbourne" have always appeared to be identical, but "P.R.8" differed significantly in being lethal for a majority of mice immunized with "Melbourne" egg virus.

Studies on the antibody content of human sera, which will be reported in subsequent papers, indicate that in the sera of young children at least the antibodies responsible for inactivating the two human virus types may be as sharply distinct as they are in convalescent ferret sera.

SUMMARY.

1. The available strains of influenza virus can be divided into three sharply defined antigenic groups, of which two comprise strains of human origin, the third containing the virus of swine influenza.

2. The strains "Melbourne", "Philadelphia", and "P.R.8" form one group (New World type) and the strains "W.S.", "B.H.", "Moscow", and "Leningrad" another (Old World type).

3. This differentiation has been shown (a) by the titration of convalescent ferret sera against representative strains on the chorioallantois and by intranasal inoculation in mice, (b) by active immunity tests in mice and ferrets, and (c) by antibody absorption methods.

ACKNOWLEDGMENT.

I am indebted to Miss D. Lush for carrying out most of the egg membrane titrations against swine influenza virus.

REFERENCES.

Burnet, F. M. (1936a): Brit. J. exp. Path., 17, p. 282.
Burnet, F. M. (1936b): Austral. J. exp. Biol., 14, p. 241.
Burnet, F. M. (1936c): *Ibid.*, 14, p. 247.
Burnet, F. M. (1937): Brit. J. exp. Path., 18, p. 37.
Burnet, F. M., Geogh, E. V. and Lush, D. (1937): Austral. J. exp. Biol., 15, p. 231.
Laidlaw, P. P., Smith, W., Andrewes, C. H. and Dunkin, G. W. (1935): Brit. J. exp. Path., 16, p. 275.
Magill, T. P. and Francis, T. (1936): Proc. Soc. exp. Biol., N.Y., 35, p. 463.
Smorodintseff, A. A., Drobyshevskaya, A. I. and Shishkina, O. I. (1936): Lancet, 2, p. 1383.

INFLUENZA VIRUS ON THE DEVELOPING EGG

6. COMPLEMENT FIXATION WITH EGG MEMBRANE ANTIGENS

by

DORA LUSH AND F. M. BURNET[1]

(From the Walter and Eliza Hall Institute, Melbourne).

(Submitted for publication 7th September, 1937.)

Wilson Smith (1936) described positive complement fixation tests with sera from immunized animals or human beings, using antigens prepared from tissues infected with influenza virus. He found that while the majority of sera showed a correlation between the power to fix complement with influenza antigens and the power to inactivate virus, exceptions were relatively common. It was found impossible to differentiate between antibodies against human and swine strain viruses by means of the complement fixation test.

Fairbrother and Hoyle (1937) also reported complement fixation tests with human sera, using a technique differing in several respects from that used by Smith. Their antigen was prepared from dried consolidated mouse lungs, and they used a short period of fixation at 37° C. instead of the ice box technique. Their results indicated that to some extent at least the complement fixing titre of a serum was an index of immunity to influenza.

Our own work has been directed first to determine the suitability of egg membrane virus as an antigen for complement fixation tests, and second, to provide further data for the comparative study of the antibody content of experimental and human sera against influenza virus. The interpretation of influenzal antibodies in human sera has already become a difficult problem, which is likely to be solved only if all the available methods of serological analysis are applied.

Preliminary work showed that infected chorioallantoic membranes provided very satisfactory antigens for complement fixation. The membranes from embryos infected with the "Melbourne" strain were removed at two days after inoculation, just before or after the death of the embryo. Some experiments were made with an antigen prepared from the whole embryo, but although this gave satisfactory results, it was more turbid and liable to precipitation than that prepared only from the infected membrane. We may mention here that good antigens for complement fixation with appropriate anti-sera can also be prepared from

[1] Work supported by a grant for research on virus diseases from the Rockefeller Foundation and the Commonwealth Government Department of Health.

membranes infected with myxomatosis virus (Lush, unpublished). Myers and Chapman (1937) have recently stated that satisfactory vaccinia virus antigens for this purpose can also be obtained from chorioallantoic membrane lesions.

TECHNIQUE.

Antigens. All antigens were made from egg-adapted strains of virus. Eggs were inoculated with undiluted stock emulsions according to the technique previously described (Burnet, 1936) and membranes removed at appropriate intervals—for strains "Melbourne" and "W.S.", two days, and for "Swine" strain, three days after inoculation. Membranes were washed in saline and allowed to drain in a tilted petri dish before being ground with quartz powder and emulsified in a small volume—about 1 c.c. per membrane—of saline. The emulsions were spun in the angle centrifuge (3,000 r.p.m.) for 30 minutes, and merthiolate (1/20,000) added.

A small amount of flocculer precipitate frequently settled out in three or four days and was removed by spinning. In the early stages of this work all batches of antigen were titrated in detail with complement, but as no anti-complementary activity was ever detected, this practice was discontinued. There is very little variation in the strength of different batches of any antigen, and it has been convenient to make small amounts weekly.

Sera. All sera were freshly inactivated before use (56°C. for 20 minutes). Serum K., a high titre adult human serum, was used as a standard in all tests. Except where otherwise stated, the ferret sera used were from *convalescent* ferrets, i.e. animals which had received intranasal inoculations of virus only. In the dilutions used for experiment these sera did not inhibit haemolysis by 1½ M.H.D. of complement.

Complement. Fresh guinea-pig serum was titrated each day, and a dilution made so that 0·1 c.c. (2 drops from a standard pipette) contained 3 M.H.D.

Sensitized red cells. The test dose consisted of 0·1 c.c. of a sensitized 3 p.c. suspension of sheep erythrocytes containing 5 M.H.D. of haemolytic amboceptor per c.c.

The test. Doubling dilutions of each serum were prepared and the test set up as follows: Saline, 0·1 c.c. (2 drops), serum, 0·1 c.c., complement, 0·1 c.c.. antigen, 0·1 c.c. The reagents were added to the tubes in the order given, mixed thoroughly by shaking, and placed in the ice box over night (usually 18 hours). The sensitized cells were then added, the mixtures shaken and placed in the water bath at 37°C. for half-an-hour, and shaken frequently. The results were read immediately after removal from the water bath. Controls always included the lowest dilution of each serum and the lowest dilution of each antigen used with 1½ M.H.D. complement, also 1½ M.H.D. complement with saline.

In discussing the results, references to dilution of antigen or serum are in terms of final dilution in the completed test, i.e. five times the dilution at which the reagents were added.

Optimal Antigen Concentrations for Different Types of Immune Sera.

Very early in our work it was found that convalescent ferret sera and high titre human sera showed a sharp difference in the concentration of antigen which gave fixation to the highest serum dilution. This optimal concentration of antigen was much higher with ferret sera than with human sera. In order to avoid large tables, we have expressed the results of experiments in which various dilutions of antigen and antiserum were used against 3 M.H.D.'s of complement in the form of small diagrams such as those shown in Fig. 1. The shaded regions indicate mixtures in which fixation is complete: where such areas are enclosed by a firm line

an end point has been obtained. If partial fixation is present, the line indicating the serum dilution end point has been placed at an intermediate position.

It will be seen that the adult human sera show an optimal fixation at an antigen dilution of 1 : 100, while convalescent ferret sera give an optimal value at 1 : 10 or 1 : 25. The examples given are characteristic of seven ferret sera and twenty adult human sera, for which data are available.

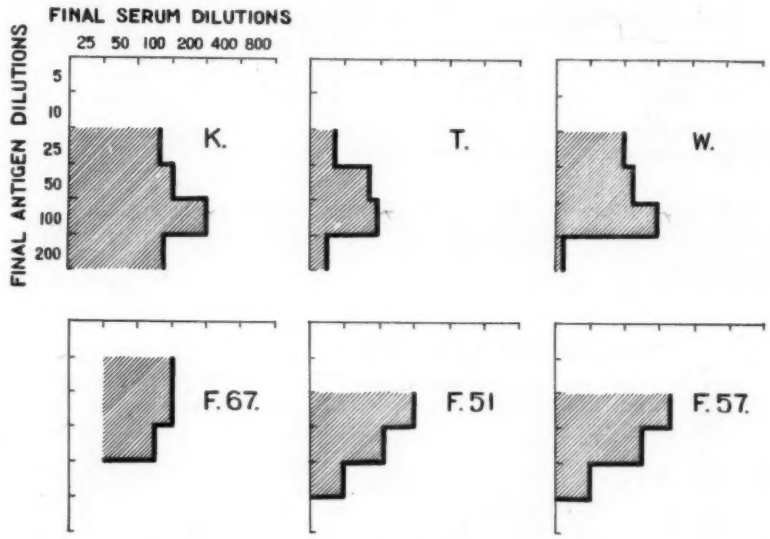


Figure 1. Complement fixation of human (K., T., W.) and convalescent ferret (F.67, F.51, F.57) sera with "Melbourne" antigen.
In all diagrams the shaded areas show complete fixation of 3 M.H.D.'s complement with the mixtures containing the final dilutions of serum and antigen shown.

Assuming that the difference between the two types of serum was related to the repeated clinical or subclinical infections with different strains of influenza virus which adult human beings have undergone, an attempt was made to imitate such conditions in ferrets. Two ferrets, F.59 and F.60, were first immunized by intranasal inoculation of egg virus, "Melbourne" strain, on 22/3/1937. They had no temperature reaction, but developed antibody showing against "Melbourne" egg virus reductions to 0·04 p.c. and 0·03 p.c. respectively. The two ferrets were then re-inoculated intranasally, F.59 with strain "P.R.8" and F.60 with strain "Philadelphia". There was no temperature reaction, although controls reacted normally. Thereafter each ferret received three subcutaneous inoculations with "Melbourne" strain (mouse virus) spread over three months. At

the end of this period the titres against "Melbourne" egg virus were F.59, 0·0032 p.c.; F.60, 0·0035 p.c. On 12/7/1937 both were given strain "W.S." and both gave a typical diphasic temperature reaction. A final bleeding was made three weeks later. The sera then showed the following titres on the egg membrane against each type of virus:

TABLE 1.

Activity of Final Sera of F.59 and F.60 Against Egg Virus Strains.

	Melbourne.	W.S.	Swine.
F.59	0·016%	0·0011%	2·1%
F.60	0·021%	0·007%	1·7%

The activity of sera is expressed as the percentage to which the count of specific foci is reduced by mixing with an equal volume of undiluted serum.

The results of complement fixation tests on these sera are shown in Fig. 2. It will be seen that there is a close approach to the type of reaction found with adult human serum.

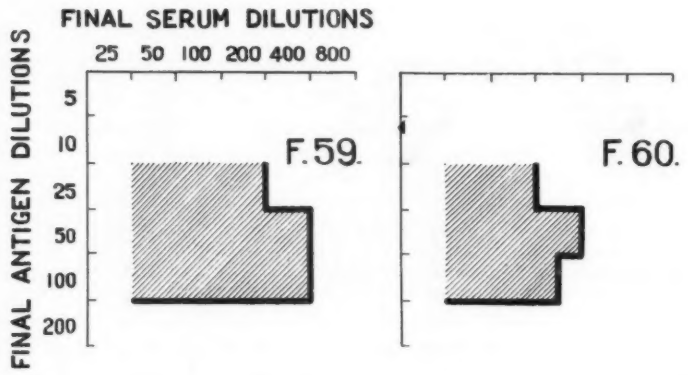


Figure 2. Complement fixation with "Melbourne" antigen of sera from two hyper-immunized ferrets to show change in optimal antigen concentration.

A number of children's sera have been tested, including a few with high neutralizing activity. Sera from eight children aged three to nine years were tested with "Melbourne" antigen at optimal dilution for adult sera (i.e. 1 : 100). None showed any fixation at the lowest serum dilution, 1 : 12·5. A repetition with more concentrated antigen showed that three of the sera had complement fixing

power of the type shown in Fig. 3. It will be seen that, although they are much weaker, the sera give diagrams of the same general type as convalescent ferret sera.

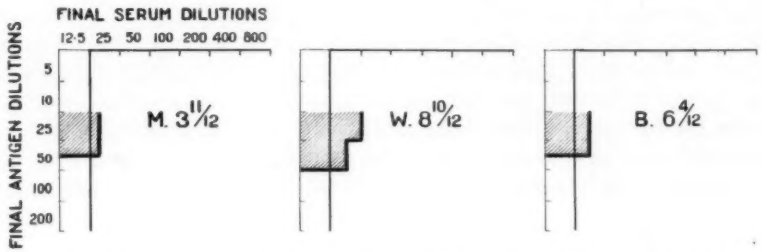


Figure 3. Complement fixation with sera from children against "Melbourne" antigen.

When similar tests are made with swine type antigens, the difference between the two types of sera is not seen. Fixation to the highest serum dilution occurs with more concentrated antigen, both for adult human and for convalescent ferret sera (Fig. 4).

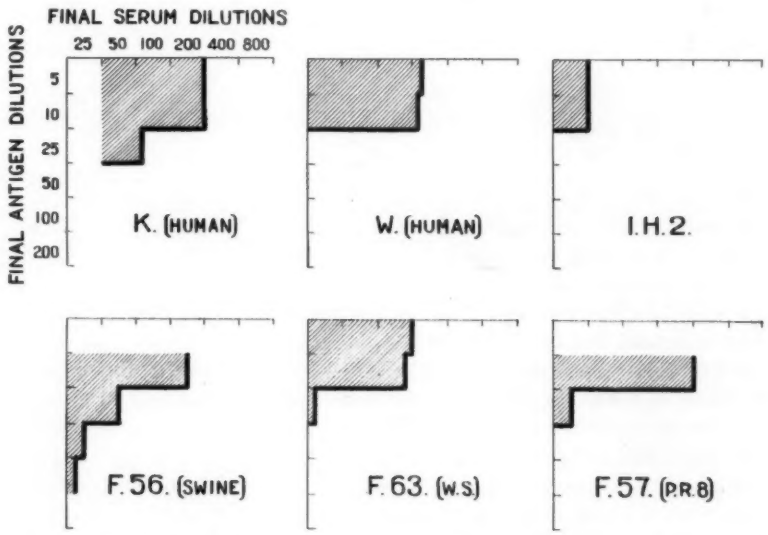


Figure 4. Fixation with "Swine" antigen showing similarity of adult human sera (K. and W.) and convalescent ferret sera.

Complement Fixation with Human Sera.

We have not found the complement fixation test of much value in the study of human sera. We can confirm Smith's observation that there is a general correlation between the inactivating power of a serum and its ability to fix complement. All the unusually active sera we have tested have given positive complement fixations, and all those which contain no demonstrable neutralizing antibody have failed to fix complement. Intermediate sera, however, show very little correlation between the two tests. There is much difficulty also in obtaining reproducible results with such sera which make up the majority of adult sera at the present time; fixation usually occurs only in the first tube (1 : 25 serum), and on retest may be absent.

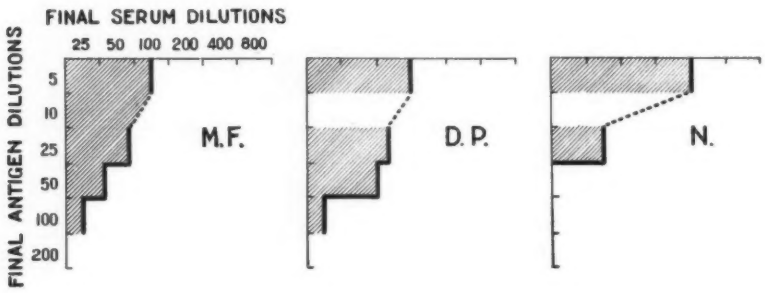


Figure 5. Complement fixation by three adult human sera with nonspecific egg membrane antigen (myxomatosis virus).

A further difficulty is the not infrequent occurrence of fixation with control egg membrane antigen containing no influenza virus. We have tested 23 adult human sera with an antigen prepared from egg membranes infected with the virus of rabbit myxomatosis. Four showed fixation to about the same degree as was obtained with "Melbourne" and "Swine" influenza antigens. Fig. 5 shows the detailed results with three of these sera.

A series of 49 human sera have been tested against both "Melbourne" and "Swine" strains, using egg membrane antigens diluted 1 : 100 and 1 : 10 respectively. With hardly an exception, the results were similar with each antigen, the difference being generally slightly in favour of the "Swine" antigen. Of these 49 individuals 2 only showed complete fixation with "Melbourne" antigen at serum dilutions of 1 : 100; 6 others did so at 1 : 50, 13 at 1 : 25 and 28 failed to give complete fixation at 1 : 25. These figures do not include the four human sera which showed nonspecific fixation with a myxomatosis virus antigen.

Results with Ferret Sera.

All convalescent ferret sera which have been examined show complement fixing power with antigens of each of the three antigenic types of influenza virus. To date, only a limited number have been tested with "W.S." type antigens, but all those available have been tested with "Melbourne" and "Swine" antigens. Table 2 gives a summary of the reactions obtained. The figures given are the final dilutions of serum with which complete fixation of 3 M.H.D.'s of complement is given, using the optimal antigen concentration 1 : 25. Where partial fixation occurred, a suitable value is interpolated between the dilutions actually tested, which, as a routine, were doubling dilutions from 1 : 12·5 or 1 : 25 to 1 : 200 or 400.

TABLE 2.

Complement Fixation with Ferret Sera.

Ferret.	Immunized with	Complement fixation with		
		Melbourne.	W.S.	Swine.
F.51	"Melbourne"	100		40
F.67	"	200	150	125
F.57	"P.R.8"	200	100	100
F.58	"Philadelphia"	150		35
F.63	"W.S."	125	200	125
F.65	"Leningrad"	75		30
F.55	"Swine"	10	10	60
F.56	"	55		150

It will be seen that there is some indication of specificity in that the titre is always highest with the homologous antigen, but except in the case of serum F.55, the differences in titre are trivial compared with the differences in virus inactivating power shown by the same sera (Burnet, 1937). None of the ferret sera gave any nonspecific reactions with antigens prepared from egg membrane lesions of an unrelated virus (rabbit myxomatosis).

DISCUSSION.

The results obtained in regard to the different types of immune serum have been consistent, but we can offer no detailed interpretation of their significance. As judged by the results with human type antigens, the sera fall naturally into two groups:

(1) Those obtained from donors subjected to one or a small number of antigenic stimuli—children and convalescent ferrets. These sera require a high concentration of antigen to show optimal complement fixation.

(2) Sera from donors subjected to repeated antigenic stimuli not necessarily all corresponding to one type of virus—adult human beings and hyperimmunized

animals. For such sera a much lower antigen concentration is required for optimal fixation.

It is well known that the quality of antibody present in the blood changes when an animal is repeatedly re-inoculated with a single antigen. Antibody produced as a result of a few closely-spaced inoculations is generally more specific than that obtained when inoculations have been continued for months. Heidelberger and Kendall (1935) have carried out a detailed examination of the precipitin reaction with crystalline egg albumen, using sera obtained at successive stages of immunization from the same rabbit. They concluded that antibody developed in the later stages of immunization is reactive with a larger number of chemically distinct groupings on the antigen molecule than the antibody formed in the early stages. Browning and Wilson (1911) noted that haemolytic antibody produced early in the course of immunization was relatively deficient in its power of binding complement. After repeated injections the complement binding power was much greater and remained relatively high, even when the actual antibody content of the serum as measured by the haemolytic dose had fallen considerably.

The complexity of the various reagents used in the test makes it impossible to offer any simple interpretation of this difference in optimal antigen concentration with the two types of serum. The position becomes even more difficult when we endeavour to correlate the results with "Swine" influenza virus antigen. With this antigen all types of sera seem to show best fixation with the highest concentration of antigen. This may to some extent merely indicate that our "Swine" type antigens contain much less actual antigen than the human type ones, but the cross reactions with ferret sera (Table 2) are strongly opposed to this view. If we take the result on its face value, it would indicate that antibody of any type which is capable of reacting at all with "Swine" virus behaves as if it were of high avidity. Such a point of view would lead to important consequences in regard to the interpretation of the high content of antibody capable of neutralizing "Swine" type virus in most adult human sera. This problem will be discussed in a later paper of this series, but our present point of view may be briefly indicated as in favour of the hypothesis that the reagent in human sera which inactivates "Swine" type virus is "low grade" antibody produced by repeated re-infections with one or both of the human type strains, not by a hypothetical strain of "Swine" type antigenic structure.

SUMMARY.

1. Egg membrane lesions produced by any of the three antigenic types of influenza virus provide satisfactory antigen for complement fixation tests.

2. The optimal concentration of antigen for fixation varies according to the type of serum being tested. Adult human sera and sera from hyperimmunized

animals show a low optimal concentration of human type antigen; sera from young children and convalescent ferrets a high optimal concentration.

3. All sera are alike in needing a high concentration of "Swine" type antigen to give optimal fixation.

4. Cross tests with convalescent ferret sera show best fixation with the homologous strain, but the degree of specificity is trivial to that shown in neutralization tests.

5. Human sera usually fix complement to about the same degree with both human ("Melbourne") and "Swine" type antigens.

REFERENCES.

Browning, C. H. and Wilson, G. H. (1911): J. Hyg., 11, p. 208.
Burnet, F. M. (1936): Sp. Rep. Ser. Med. Res. Coun., Lond., No. 220.
Burnet, F. M. (1937): Austral. J. exp. Biol., 15, p. 369.
Fairbrother, R. W. and Hoyle, L. (1937): J. Path. Bact., Lond., 44, p. 213.
Heidelberger, M. and Kendall, F. E. (1935): J. exp. Med., 62, p. 697.
Myers, R. M. and Chapman, M. J. (1937): Amer. J. Hyg., 25, p. 16.
Smith, W. (1936): Lancet, 2, p. 1256.

PHYSIOLOGICAL ONTOGENY IN PLANTS AND ITS RELATION TO NUTRITION

3. THE EFFECT OF NITROGEN SUPPLY ON THE DRIFTING COMPOSITION OF THE LEAVES.

by

A. H. K. PETRIE

(From the Waite Agricultural Research Institute, University of Adelaide[1]).

(Submitted for publication 22nd October, 1937.)

INTRODUCTION.

In the first paper of this series (Ballard and Petrie, 1936) it was pointed out that the growth and development of an organism is the result of a number of ontogenetic processes, among which complex interrelations exist. The interpretation of these interrelations, and of the manner in which the processes are integrated to produce the living plant, is the fundamental problem in the study of growth. Following the schema of Needham (1933), ontogenetic processes may be classified as follows:

1. *Growth.* This includes firstly change in size and weight. We measure, however, not only the change in the dry weight, but also the change in the weights of various of the components of the dry matter; and the measurement of the relation of the weights of these to the total dry weight enables us to observe changes in the chemical composition of the plant and its parts. The water content may also be classified here.

2. *Changes in Structure and Organization.* This includes differentiation or increase in complexity and organization, morphological changes of all kinds, and changes in the relative proportions of organs. Of some of these processes we lack means of measurement, although we can determine the drifts in the ratios of the dry weight of the various parts to that of the total plant.

3. *Metabolism.* This includes the whole category of chemical reactions proceeding in the organism and resulting in the formation of new material.

The processes of the first two groups can be regarded as the expression of those of the third, but interactions and interrelations occur among the processes of all

[1] This represents one of a series of investigations carried out at the Waite Institute and financed co-operatively by the Carnegie Corporation of New York, the Council for Scientific and Industrial Research, and the University of Adelaide.

the groups; thus changes in structure and composition can influence the subsequent course of the metabolic reactions that caused them.

In the first paper, and in the second of this series (Williams, 1936), were described the drifts that occur in the dry weight of the plant and its parts, and in various quantities derived therefrom, when different amounts of nitrogen and phosphorus are supplied. It was shown how the rate of accumulation of dry matter, a process of the first group, is the product of the unit leaf rate, a measure of metabolism, and the leaf weight ratio, a measure of morphological development. In this third paper and others to follow, another aspect of the growth of the plant will be considered, namely the drifts in the amounts of various constituents in the organs of the plant and the effects on the drifts of variation in the nutrient supply. Such effects are not only of interest in themselves, as adding to our knowledge of the relation of the growth of the plant to the environment, but are of value since they aid in the understanding of the mechanism of and interrelations among ontogenetic processes. This paper is limited to the consideration of the absolute and relative amounts of protein and non-protein nitrogen, total phosphorus, and water in the leaves for the two experiments described in paper 1, where wheat and Sudan grass were grown with varying nitrogen supply. It will be shown how the drifts in these chemical attributes of the plant are also dependent upon structural change, and on processes in metabolism and the changing rates thereof. The interpretation of the drifts, however, is not considered exhaustively in the present paper, since this would involve drawing more largely on other data existing in the literature than is feasible at the present time. The data given here will be utilized again in a subsequent paper when they come to be considered in their relation to the unit leaf rate. It must be realized that the conclusions drawn here apply only to the particular levels at which the nutrients were supplied.

In the case of the wheat experiment a considerable amount of the analytical work was carried out by Dr. L. A. T. Ballard prior to his departure from this Institute. The author regrets that Dr. Ballard was unable to complete the investigation as was originally intended.

ANALYTICAL METHODS.

The replicates of dried leaf material of the two experiments described in paper 1 were combined according to harvest and treatment, and ground in a mill to pass a 1 mm. sieve. The powder was then sampled for estimation of the total and protein nitrogen and total phosphorus. Mean values for the results of these estimations, which were performed in duplicate, are given in the tables.

Total nitrogen was estimated by the Kjeldahl and micro-Kjeldahl methods.

In estimating protein nitrogen the dried material was extracted by bringing to the boil in water, placing on the water-bath for ten minutes, and then allowing to cool to 40 °C. The protein was then precipitated with a solution of 4 p.c. tannin and 1 p.c. hydrochloric acid. The precipitate was filtered and washed with 1 p.c. tannin solution and the nitrogen estimated by the Kjeldahl method.

The fraction termed soluble nitrogen represents the difference between total and protein nitrogen.

Phosphorus was estimated by the method of Zinzadzé (1931).

Water contents were calculated from the differences between fresh and dry weights of each of the replicates of leaf material.

DESCRIPTION OF DRIFTS.

In the following paragraphs will be described some of the more outstanding features of the drifts in the composition of the leaves in the two plants used for the experiments. The nitrogen treatments were as follow:

Wheat.

I	1	gm.	$NaNO_3$	per	pot.
II	3	,,	,,	,,	,,
III	6	,,	,,	,,	,,
IV	10	,,	,,	,,	,,

Sudan Grass.

I	0·75	gm.	$NaNO_3$	per	pot.
II	2·25	,,	,,	,,	,,
III	4·50	,,	,,	,,	,,

Other experimental details are given in paper 1.

Total Leaves.

ABSOLUTE DRIFTS.

Wheat (Table 1; fig. 1).

Protein Nitrogen.

Determinations were commenced at harvest 6, at which point the values with treatments II to IV considerably exceed that with I, while that with treatment IV does so to a less extent, as is the case with the dry weight values. By harvest 7 treatment effects are much more marked, after which the values tend to converge as they fall. Examination of the curves shows that the maximum has been delayed by treatment, at least as between I and the remaining treatments. The maxima are earlier than the corresponding maximum dry weights, which also occur later the higher the treatment.

Soluble Nitrogen.

The picture is similar to that for protein nitrogen, except that there is less evidence for delay of the maximum, which occurs with each treatment at harvest 7.

Total Phosphorus.

As in the case of protein nitrogen, there is an increase in the values with treatment, although it is of smaller magnitude than in the case of protein nitrogen. Treatment IV has no greater effect than III until harvest 7. The maxima are all at harvest 7.

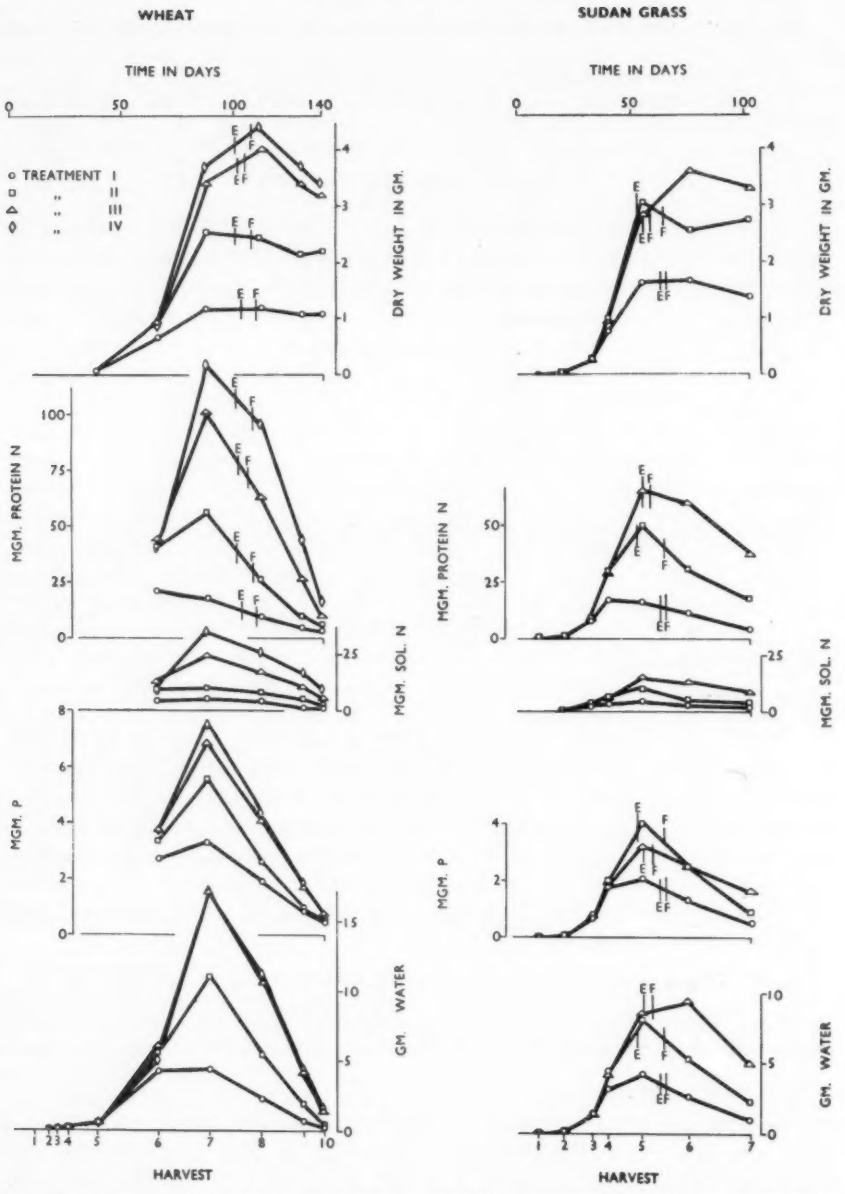


Fig. 1. Dry weight of and absolute amounts of constituents of total leaves per plant. From data of Tables 1 and 3. In this and subsequent figures, E indicates times of exsertion of inflorescences and F, time of flowering.

Water.

At harvests 2 and 3 the absolute water content falls progressively with treatment. At harvest 4 an increase is shown with treatment II as compared with treatment I and at harvest 6 with III as compared with II. The characteristic depression with high treatment is thus seen as in the dry weight. After harvest 5 effects become marked with treatments II and III, but there is no difference in effect between treatments III and IV as in the case of the other constituents. Maximum is attained with treatment I between harvests 6 and 7, and with the remaining treatments at harvest 7; it is possible therefore that the higher treatments have delayed the attainment of the maximum. Apart from the absence of effect between treatments III and IV the picture resembles that for protein nitrogen.

Sudan Grass (Table 3; fig. 1).

Protein Nitrogen.

The values with treatment III are less than those with I and II till after harvest 4, whereas the depression in dry weight persists till after harvest 5. Treatment effects become large after harvest 4. The maximum again appears to be delayed by treatment, and except with treatment II is earlier than the time of attainment of maximum dry weight. The decline is less rapid than in the wheat experiment, and within the time of the experiment convergence is less marked.

Soluble Nitrogen.

The picture is similar to that for wheat.

Total Phosphorus.

Depression occurs with treatment III until between harvests 5 and 6, which corresponds to the depression in dry weight. The maxima are at harvest 5, whereas with treatment I the protein nitrogen maximum was possibly earlier.

Water.

The picture resembles that for wheat, with a considerable delay in maximum with treatment III, and less marked convergence in senescence.

RELATIVE DRIFTS.

Wheat (Table 2; fig. 2).

Protein Nitrogen.

There is a continuous decline with time in protein nitrogen content from the commencement of the data. There is an increase with nitrogen supply, although not in proportion to the magnitude thereof. At harvest 6 treatment II has pro-

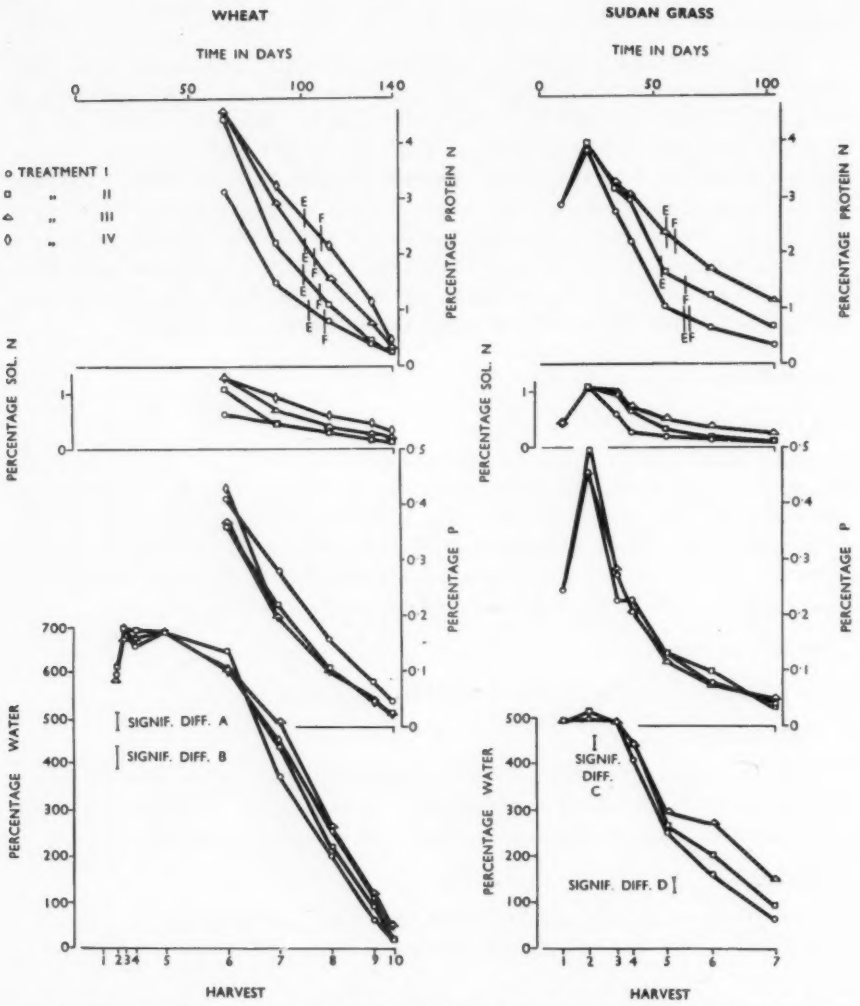


Fig. 2. Amounts of constituents of total leaves in gm. per 100 gm. dry matter. The significant differences are 2·828 × the standard error. A is for the standard error of means of 5 (all points except those to which B is applicable); B is for means of 3 (treatment IV, harvests 3, 4 and 6); C is for means of 6 (harvests 2–3); D is for means of 5 (harvests 4–7). From data of Tables 2 and 4.

duced much larger plants than treatment I; but effects of the higher treatments do not appear till later. There is probably a saturation capacity of the tissues for protein at a given stage in their history, which would account for the effects of the

high treatments not appearing until the values with the lower treatments have fallen through their normal ontogenetic drift. All the curves tend to converge at the end of the growing period. This is essentially the picture given by Gregory (1937) for the effect of increasing nitrogen supply on the nitrogen content of the whole plant in barley.

Soluble Nitrogen.

This picture is similar to that for protein nitrogen, except that the curve for treatment II becomes coincident with that for treatment I at a comparatively early stage in the history.

Total Phosphorus.

Comparison of the graph with those of fig. 3, paper 1, shows that in general the percentage phosphorus in the leaves is less the greater the size of the plant, although the absolute amount is greater.

Water.

Analysis of variance was performed on the replicated data for this quantity. Time, treatment and interaction were significant, and the standard error is given in the Table.

An early maximum is followed by a progressive fall lasting for most of the growing period. Depression is suggested with treatments III and IV at harvest 4, but is insignificant. After harvest 6 the water content increases with treatment from I to III and is depressed again with treatment IV; these effects can be shown to be significant by combining the data of harvests 6–10. Probably depression occurred with treatment IV throughout the whole history, although this is not revealed by the data.

Sudan Grass (Table 4; fig. 2).

Protein Nitrogen.

There is a rise to a maximum at harvest 2, at which there is probably no significant effect of treatment. The subsequent decline is more rapid the lower the treatment, so that the protein content becomes greater the greater the treatment. The convergence shown in the wheat data does not occur here, although it might have appeared if observations had been continued later.

Crowther (1934) concluded from his investigations on the cotton plant that a certain constant nitrogen content represented a critical level at which dying off of old leaves overtook production of new leaves; in neither of the present experiments, however, is this the case, since the maximum dry weight occurs at different nitrogen contents.

Table 1.

Absolute Amounts of Protein and Soluble Nitrogen, Total Phosphorus, and Water in the Leaves, Exp. 1 (Wheat).*

	Treatment I.				Treatment II.				Treatment III.				Treatment IV.			
Harvest	Prot. N.	Sol. N.	Total P.	Water	Prot. N.	Sol. N.	Total P.	Water.	Prot. N.	Sol. N.	Total P.	Water.	Prot. N.	Sol. N.	Total P.	Water.
2				0·0565				0·0544				0·0524				0·0488
3				0·128				0·122				0·118				0·102
4				0·161				0·200				0·188				0·140
5				0·436				0·412				0·433				—
6	20·8	4·1	2·72	4·24	41·5	10·1	3·38	5·64	45·4	12·9	3·66	5·98	39·4	11·3	3·69	5·10
7	17·4	5·4	3·28	4·33	55·9	11·4	5·57	11·1	101·5	24·7	6·86	16·9	122·2	35·3	7·50	17·0
8	9·76	3·7	1·87	2·35	26·9	7·6	2·60	5·51	63·4	17·6	4·01	10·5	96·1	27·3	4·34	11·3
9	4·29	1·86	0·864	0·651	10·0	5·1	0·968	1·96	26·3	10·4	1·70	4·02	43·6	16·8	1·83	4·27
10	2·65	1·42	0·482	0·202	5·81	3·7	0·528	0·441	11·3	6·3	0·704	1·36	16·6	11·4	0·787	1·67

* Amount of water in grammes, of remaining entities in milligrammes, per plant.

Table 2.

Percentage Amounts of Protein and Soluble Nitrogen, Total Phosphorus and Water in the Leaves, Exp. 1 (Wheat).*

	Treatment I.				Treatment II.				Treatment III.				Treatment IV.			
Harvest	Prot. N.	Sol. N.	Total P.	Water.	Prot. N.	Sol. N.	Total P.	Water.	Prot. N.	Sol. N.	Total P.	Water.	Prot. N.	Sol. N.	Total P.	Water.
2				595				613				582				610
3				698				698				670				696
4				655				695				672				668
5				686				685				683				—
6	3·14	0·61	0·41	643	4·43	1·08	0·36	602	4·59	1·30	0·37	605	4·60	1·31	0 43	599
7	1·49	0·46	0·28	371	2·21	0·45	0·22	438	2·96	0·72	0·20	493	3·26	0·94	0·20	454
8	0·82	0·31	0·16	197	1·10	0·31	0·11	219	1·58	0·44	0·10	262	2·15	0·61	0·10	252
9	0·40	0·17	0·080	60	0·47	0·23	0·045	91	0·77	0·31	0·050	118	1·17	0·45	0·049	114
10	0·25	0·13	0·045	19	0·26	0·17	0·024	20	0·35	0·20	0·022	42	0·49	0·33	0·023	49

* The values for water are the amounts associated with 100 gm. dry matter.

Water data: S.E. for means of 3 (treatment IV, harvests 3, 4 and 6) = ± 50·1.
S.E. for means of 5 (remaining data) = ± 38·7.

TABLE 3.

Absolute Amounts of Protein and Soluble Nitrogen, Total Phosphorus, and Water in the Leaves, Experiment 2 (Sudan Grass).*

Harvest	Treatment I.				Treatment II.				Treatment III.			
	Prot. N.	Sol. N.	Total P.	Water.	Prot. N.	Sol. N.	Total P.	Water.	Prot. N.	Sol. N.	Total P.	Water.
1	0.0667	0.0101	0.00559	0.0114	0.0667	0.0101	0.00559	0.0114	0.0667	0.0101	0.00559	0.0114
2	0.964	0.28	0.115	0.129	0.934	0.24	0.117	0.119	0.770	0.207	0.0882	0.099
3	7.95	1.73	0.645	1.40	9.35	2.81	0.791	1.46	8.46	2.63	0.725	1.26
4	17.01	2.16	1.75	3.18	29.80	6.60	2.04	4.41	28.49	6.78	1.93	4.15
5	16.46	3.28	2.10	4.19	50.33	10.37	4.00	8.01	66.83	15.23	3.24	8.43
6	10.96	2.56	1.27	2.65	31.23	5.12	2.53	5.24	61.74	12.98	2.64	9.47
7	4.64	1.87	0.542	0.89	17.84	3.73	0.952	2.36	37.62	9.24	1.62	4.92

*Amounts of water in grammes, of remaining entities in milligrammes, per plant

Soluble Nitrogen.

The picture is similar to that for protein nitrogen except that, as in the wheat experiment, the curves for treatments I and II coincide some distance before the end of the history.

Total Phosphorus.

At harvest 2, the position of the maximum, it is possible that the size of the plant significantly affects the percentage phosphorus content, but thereafter treatment does not appear to have any effect.

Water.

Analysis of variance was again performed on the water content data, time, treatment and interaction being significant. The standard error is given in the Table.

The water content remains approximately constant till harvest 3; rapid decline then sets in, and the value becomes greater the greater the treatment.

THIRD LEAF OF PRIMARY SHOOT.

Wheat (Tables 5 and 6; figs. 3 and 4).

ABSOLUTE DRIFTS.

Total Nitrogen.

The picture is similar to that for the total leaves, except that it is condensed in time. The incidence of the maximum does not appear to be affected by treatment. There is a very marked increase with treatments II and III, and some suggestion that the nitrogen content is reduced again with treatment IV.

Water.

The time drift follows that of the dry weight, but the treatment effects are slight until harvest 7. There is a suggestion of depression with the higher treatments at the earlier harvests.

RELATIVE DRIFTS.

Total Nitrogen.

There is a marked increase from the values obtained with I to those obtained with the remaining treatments, but other treatment effects are slight.

Water.

Analysis of variance was performed on the replicated data; time, treatment and interaction are significant.

There is a prolonged maximum followed by rapid fall in senescence with treatment I. The variability of the individual pot values is high, and no significant effects of treatment can be seen until harvest 7. Here there is progressive increase with treatments I to III, whereas the values with treatments III and IV are insignificantly different; there is thus a marked delay in senescent fall with the higher treatments.

TABLE 4.

Percentage Amounts of Protein and Soluble Nitrogen, Total Phosphorus and Water in the Leaves, Experiment 2 (Sudan Grass).*

	Treatment I.				Treatment II.				Treatment III.			
Harvest	Prot. N.	Sol. N.	Total P.	Water.	Prot. N.	Sol. N.	Total P.	Water.	Prot. N.	Sol. N.	Total P.	Water.
1	2·90	0·44	0·243	491	2·90	0·44	0·243	491	2·90	0·44	0·243	491
2	3·84	1·09	0·456	512	3·99	1·03	0·500	509	3·87	1·04	0·443	496
3	2·76	0·60	0·224	490	3·19	0·96	0·270	498	3·28	1·02	0·281	490
4	2·20	0·28	0·227	406	2·98	0·66	0·204	441	3·07	0·73	0·208	444
5	1·01	0·20	0·129	253	1·65	0·34	0·131	268	2·37	0·54	0·116	299
6	0·664	0·156	0·077	160	1·22	0·20	0·099	205	1·71	0·36	0·073	273
7	0·334	0·120	0·039	62	0·656	0·137	0·035	92	1·14	0·28	0·049	149

* The values for water are the amounts associated with 100 gm. dry weight.

Water data: S.E. for means of 6 (harvests 2–3) = ± 11·0.
S.E. for means of 5 (harvests 4–7) = ± 12·0.

DISCUSSION.

1. *The Initial Depression.*

During early growth of the plant the increases in dry weight with increases in nitrogen supply become progressively less until, beyond a certain supply, further addition of nitrogen causes reduction rather than further increase in dry weight. The larger such further addition, the longer the depression in dry weight persists. In the present experiments the initial depression in dry weight obtained with high nitrogen treatment is reflected also in the amounts of protein and soluble nitrogen, phosphorus, and water taken up by the plant. Thus actually less nitrogen at first went into the plants supplied with the larger amounts than into those supplied with less.

It may be concluded that the depression in dry weight is caused by a reduction

in the unit leaf rate over a short period immediately following the time of application of the treatments; some suggestions may, however, be made as to the chain of events leading to this reduction, and these will be derived from consideration of other data.

Goedewaagen (1929) investigated the growth of wheat plants, up to about six weeks from germination, in solutions of various concentrations of sodium

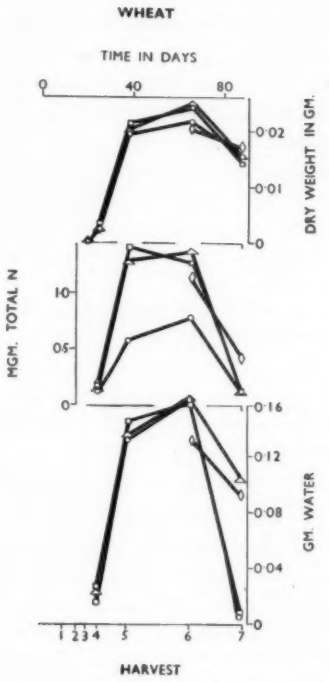


Fig. 3. Dry weight of third leaf of primary shoot and its absolute content of total nitrogen and water. From data of Table 5.

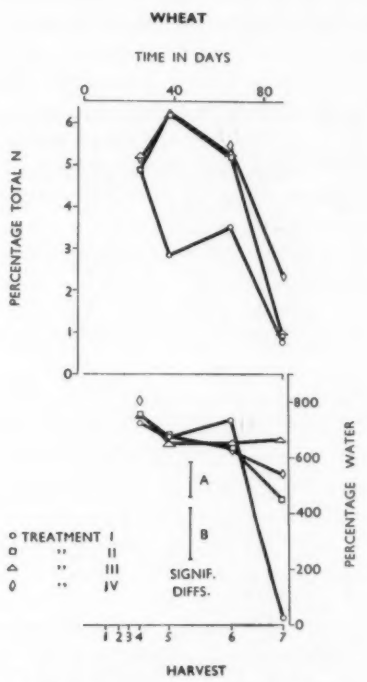


Fig. 4. Amounts of nitrogen and water in third leaf of primary shoot in gm. per 100 gm. dry matter. The significant differences are 2·828 × the standard error. A is for means of 5 (all points except those to which B is applicable); B is for means of 3 (harvests 3, 4 and 6, treatment IV). From data of Table 6.

nitrate. The results are variable, but suggest that the amounts of dry matter produced in the time increased up to a maximum with a concentration of 182 to 238 p.p.m. nitrogen, and that a decrease tended to occur with higher concentrations. The decrease was associated with lowered water content of the plants. Goedewaagen also established by experiments with sodium sulphate that the decrease was due to the NO_3^- and not to the Na^+ ion.

The depression in dry weight production by solutions of ammonium salts has been discussed by Rippel, Behr and Meyer (1933) and Rippel and Meyer (1933), who showed that similar concentrations of potassium chloride or potassium sulphate caused no such depression, and that therefore the effect could not be an osmotic one; there was furthermore no summation of effects when high concentrations of potassium sulphate and ammonium sulphate were simultaneously applied. These investigators suggest that the assimilation of the excess nitrogen absorbed takes up so much of the products of carbon assimilation that a deficiency

TABLE 5.

Absolute Amounts of Total Nitrogen and Water in the Third Leaf of the Primary Shoot, Experiment 1 (Wheat).*

	Treatment I.		Treatment II.		Treatment III.		Treatment IV.	
Harvest.	Total N.	Water.	Total N.	Water.	Total N.	Water.	Total N.	Water.
3	—	0·0013	—	0·0012	—	0·0016	—	0·0013
4	0·121	0·0174	0·171	0·0265	0·167	0·0236	0·177	0·0278
5	0·556	0·133	1·350	0·145	1·290	0·135	—	—
6	0·764	0·160	1·26	0·157	1·33	0·164	1·12	0·129
7	0·121	0·0054	0·132	0·0065	0·167	0·1055	0·416	0·0922

* Amounts of nitrogen in milligrammes, of water in grammes, per plant.

TABLE 6.

Percentage Amounts of Total Nitrogen and Water in the Third Leaf of the Primary Shoot, Experiment 1 (Wheat).*

	Treatment I.		Treatment II.		Treatment III.		Treatment IV.	
Harvest.	Total N.	Water.	Total N.	Water.	Total N.	Water.	Total N.	Water.
4	4·88	721	4·85	754	5·26	748	5·14	802
5	2·82	674	6·25	671	6·26	654	—	—
6	3·49	733	5·16	645	5·28	654	5·50	629
7	0·76	34	0·92	453	1·05	661	2·39	539

* Amounts of water in grammes associated with 100 gm. dry matter.

Water data: S.E. for means of 3 (harvests 3, 4 and 6, treatment IV) = ± 187·5.
S.E. for means of 5 (remaining data) = ± 145·1.

remains for growth; there is, however, no evidence that this could be the explanation in the present experiments where, in the leaves at all events, such excess amounts of nitrogen do not appear to have occurred.

Willis and Davis (1925) also investigated the toxic effect of various nitrogen compounds on cotton seedlings. They found the effect occurred at low osmotic pressures of the soil solution (6·29 atmospheres with ammonium sulphate, and 9·42 atmospheres with sodium nitrate)[2]; in their experiments, where reduction in dry weight occurred, the percentage nitrogen content was increased.

[2] Their contention that the osmotic pressure is different with different nitrogen compounds is hardly sufficiently well established by their data, especially in view of the admitted complication that urea gives rise to free ammonia when in contact with soil.

In another investigation in this laboratory, Petrie and Wood (1938) found that application of high concentrations of ammonium sulphate to sand cultures of young grass plants caused an immediate decrease in water content of the leaves, whereas similar applications of potassium sulphate caused a much smaller decrease; it therefore appeared that the decrease in water content was due to a specific effect of ammonia or the NH_4^+ ion. Mevius (1928, 1929) suggested that, in high concentrations of ammonium salts, the intake of considerable amounts of the ammonium radicle caused a toxic alkalinity in the sap of the roots; this might have decreased the rate at which water could be supplied to the leaves, and so caused decrease of their water content as transpiration proceeded.

We do not know whether in the present case, with high supply of nitrate ions, there would be such an extensive reduction of nitrate ions in the roots as to set up a high ammonia concentration, although there is evidence (Thomas, 1927; Nightingale, 1934; Tiedjens, 1934) that nitrate reduction can occur in roots. But in any case it seems probable that the high concentration of nitrate ions in some manner decreased the water content of the leaves. Although significant reduction of water content with high treatment was observed in only one case, it is possible that such reduction occurred for a brief period after the application of the treatments. As has been shown by Mothes (1931) and Petrie and Wood (1938), the result of decreased water content is decreased net rate of protein synthesis, so that the protein content of the leaves is reduced. In the experiments of Petrie and Wood the soluble nitrogen content was increased, but this was owing to a large accumulation of ammonia nitrogen in the leaves, which was associated with high values of amino- and amide nitrogen. In the present experiment the percentage soluble nitrogen content was not increased, so that probably such accumulations of ammonia nitrogen in the leaves did not occur in this case where nitrate was applied.

If there were temporary decreases in the contents of protein nitrogen and water, these would account for a temporary reduction in the unit leaf rate. It is apparent from fig. 8, paper 1, that with high treatments the unit leaf rate (E_W) must return to the same value as with lower treatment before any appreciable reduction in external nitrogen concentration is likely to have occurred. The mechanism of the recovery is therefore not clear at the present time.

2. *Factors Governing the Amounts of Nitrogen and Phosphorus in the Leaves.*

As Mason and Maskell (1931, 1934) have shown, import and export of nitrogen and phosphorus by the leaves are continuous processes. The position of maximum absolute content of either of these elements in the leaves represents the point at which, considering the leaves as a whole, the rates of import and export become equal; during the early portion of the growing period of the plant import preponderates, whereas later export has the greater rate.

The rates of these two processes are controlled by a nexus of factors; among these we visualize the following, so far as concerns the present experiments:

(1) Direct effect of external concentration as determining rate of intake of

nitrogen and phosphorus by the whole plant. As the nitrogen supply is increased the absolute amounts of nitrogen in the leaves are increased; the increases become smaller as the supply rises, until finally no further amount of nitrogen will enter the plant. As growth proceeds the external concentrations of both nitrogen and phosphorus fall, and this fact must contribute to the divergence in the percentage nitrogen curves in later growth, and to decline in the rate of import of both nitrogen and phosphorus by the leaves, and so to the decline in their percentage content.

(2) Rate of growth of leaves as a determinant of their capacity to take in nitrogen and phosphorus. One of the main effects of nitrogen on growth, as Crowther (1934) has pointed out, is that of initiating meristematic activity. After the initial depression in relative growth rate has passed off, increased nitrogen supply causes greater deflection of synthesized materials to leaf formation. This increased growth of leaves must contribute to the increase in the absolute amounts of nitrogen and phosphorus taken up with increased nitrogen supply. Furthermore, with increased nitrogen supply, there is more than sufficient nitrogen available to supply the extra leaf growth, so that actually the percentage nitrogen, as is seen in the present paper, becomes greater with treatment. In the case of phosphorus, where the external concentration was initially the same for all treatments, although the absolute intake is greater the greater the dry weight of the leaves, the percentage content is not greater, and in the case of the wheat experiment is less, probably owing to depletion of the external supply.

(3) Rate of growth of the other parts of the plant and the nature of their development, which affects the rate of export of nitrogen and phosphorus from the leaves. Examination of the diagrams illustrating the drifts in proportions of the parts of the plant illustrated in paper 1, fig. 12, and paper 2, fig. 6, shows that, during early growth of the plant, after the seedling stage, the leaves represent a large portion of the plant, whereas later the stems and finally the inflorescences assume increasing importance in their proportions. It may be expected that this later development of the stems and inflorescences will cause the rate of export to increase with time, until the absolute amounts of nitrogen and phosphorus begin to fall and senescence sets in. In the case of nitrogen it will tend to decrease the concentration of translocatable compounds in the leaves, and so to increase the rate of protein hydrolysis.

It was seen in the Sudan grass data that the decline in absolute protein nitrogen during the latter part of the history is less rapid than in the wheat; this is probably associated with the small inflorescence weight ratio of Sudan grass as compared with wheat (paper 1, figs. 9 and 10). The percentage protein nitrogen curves also converge in wheat in a way they do not in Sudan grass; this may be due to the fact that the inflorescence weight ratio is increased with treatment in wheat, whereas treatment effects in Sudan grass are comparatively negligible.

(4) The capacity of the root system to take up nitrogen and phosphorus. As is shown in figs. 9 and 10, paper 1, increasing nitrogen supply decreases the root weight ratio, and this may contribute to the fact that the percentage nitrogen does

not increase in proportion to the treatment. Also it is interesting to observe in the wheat experiment that, at harvest 6, when there must still have been a high external concentration of nitrogen with the highest treatments, net migration of nitrogen had already commenced from the third leaf. This may indicate that the demand for nitrogen was so great by the leaves formed later than the third that nitrogen did not enter the plant fast enough to preserve migration into the third leaf at a higher value than export therefrom. The possibility must not be overlooked, however, that other factors may have caused the initiation of net export from the third leaf.

The root weight ratio also declines with time; this may be a factor contributing to the declining percentage content of nitrogen and phosphorus. Crowther (1934) suggests that deflection of supply of nutrients from roots to inflorescences causes the roots eventually to be unable to take up more nitrogen.

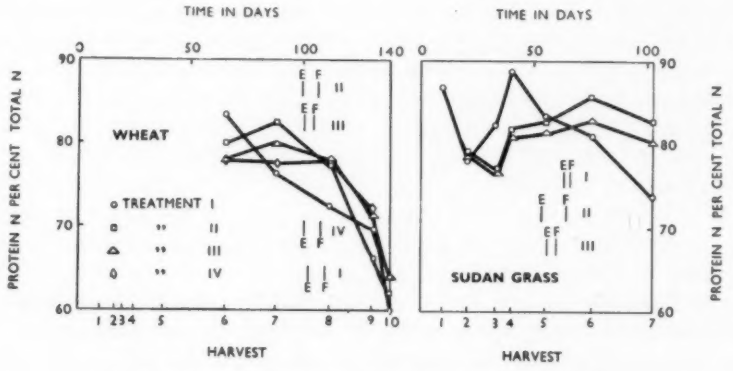


Fig. 5. Protein nitrogen as percentage of total nitrogen.

(5) Declining rate of carbon assimilation, which may lead to decreasing amount of substrate for protein synthesis and so may decrease rate of import of nitrogen.

(6) Possible internal changes in the leaves leading to decreased rate of protein synthesis or increased rate of hydrolysis. Mothes (1926, 1931) has suggested that such changes occur during the ageing of the leaf. Declining water content, which is also a characteristic of ontogeny as is seen in the present data, could produce this (Mothes, 1931; Petrie and Wood, 1938). It will be observed that absolute loss of water from the total leaves and from the third leaf is generally coincident with the commencement of net protein hydrolysis, although the Sudan grass data suggest that protein loss can commence first; but there is no case of absolute water loss preceding protein loss.

It is of interest in this connection to observe the behaviour of the protein nitrogen expressed as a percentage of the total nitrogen. The values for this quantity

are plotted in fig. 5. During the early growth of the Sudan grass the percentage falls to a minimum and then rises again to a maximum which is highest and earliest with treatment I and is later and progressively lower with the other two treatments. Over the period for which the data are available, the picture is the same for the wheat experiment, except in late senescence, where there is a considerable fall. Evidently with treatment I, where export is relatively most rapid, it is associated with a relatively greater proportion of soluble nitrogen in the leaves; but initiation of net protein hydrolysis is not necessarily associated with decreasing proportion of protein nitrogen.

Several other workers have also obtained data for the drift in protein nitrogen as percentage of the total. Smirnow (1928) found in sunflower leaves a fall, between the stages of 2–3 and 8–10 leaf pairs, from 92·5 to 91·5 p.c., and then a progressive rise to 97·1 p.c. at the "Stadium der Wachsreife der Samen"; this conforms roughly with the picture obtained with the higher nitrogen treatments in Sudan grass. In tobacco leaves Smirnow found a rise from 84·8 to 86·5 p.c. between the seedling stage and that of 5–6 leaf pairs, and then a progressive fall to 72·3 p.c. at the time of capsule formation; this is suggestive of the picture given by Sudan grass with low nitrogen treatment. In the combined leaves and stems of wheat plants growing under conditions analogous to those used in the present experiments, McCalla (1933) found a maximum shortly after flowering, and a decline thereafter to the end of the growing period.

Whether the amount of phosphorus in the leaves is influenced by changes in the rates of synthesis or hydrolysis of organic phosphorus compounds is not known at present.

(7) Establishment of a state of starvation with respect to some nutrient other than nitrogen. This might lead to hydrolysis of proteins and to breakdown of the protoplasmic system. The most probable nutrient of which the plants could have been starved is phosphorus; a differential starvation effect with respect to phosphorus could have occurred in wheat, where the percentage is lower the higher the treatment, but this is not the case in Sudan grass.

These factors are defined here as probable determinants of the rates of movement of nitrogen and phosphorus into and out of the leaves. In the case of nitrogen it is clear that there are two phenomena that may lead to net migration in senescence. One is that, as a result of decreased rate of import of inorganic nitrogen, or increased rate of export of soluble organic nitrogen, the concentration of the latter in the leaves decreases; this must lead to protein hydrolysis. The other is a change in conditions within the leaf leading to increased net rate of protein hydrolysis. It is not possible from the data available to consider more fully the part that each of the factors enumerated plays in leading to these phenomena. Consideration in the case of phosphorus is further limited by absence of knowledge of the drifts in the various forms in which it exists in the leaves.

3. *Interrelations of Amounts of Nitrogen and Phosphorus in the Leaves.*

It has been seen that the amount of phosphorus taken into the leaves from the same initial external supply is to a considerable extent determined by the size of the plants. In the wheat experiment, however, as the dry weight increases, the percentage phosphorus in the leaves becomes less, and this will be largely because of more rapid fall in external concentration of phosphorus. This is a phenomenon that has been often established in the nutrient relationships of plants, e.g. Sekera (1928), McCool and Weldon (1928), etc. Davidson (1933) also found decreased percentage phosphorus content with the application of sodium nitrate to wheat, but on the other hand the potassium content was increased. He suggests that the decreased phosphorus content is the result of increased alkalinity of the external medium; but without knowledge of the effect of the treatment on the size of the plants this suggestion cannot be regarded very favourably.

Some peculiar results are shown in the data of Blanck and Giesecke (1934), where increasing nitrogen supply to oats decreased the absolute amount of phosphorus in the leaves and stems. The plants with the highest nitrogen treatment, although eventually growing larger than those with the next lower treatment, always had a lower absolute phosphorus content. It is difficult to account entirely for these results, although it seems evident that the large inflorescences developed with the higher treatments caused a greater drain on the phosphorus in the leaves.

4. *Relation of Protein and Water Contents.*

The relation of the percentage protein to the percentage water in the leaves is shown for wheat in fig. 6; the data for Sudan grass give a similar picture. It is clear that the water content increases markedly with protein content in the later stages of growth,[3] but in adolescence the increase is considerably smaller and, in fact, at harvest 6 there is a decrease in water content with treatment. Furthermore for any given protein content the water content is less the higher the treatment, or in other words the older the plant.

Increased content of proteins may be expected to increase the water content on account of the hydration capacity of these compounds. Crist (1926) showed that, with increased nitrate supply, plants had not only greater water content but greater content of bound water as determined by the cryoscopic method. Pearsall and Ewing (1929) have also drawn attention to another way in which increased nitrogen supply may increase the water content of the plant. They found that increased nitrogen supply caused increase in the *p*H of the expressed sap; they concluded that the dissociation of the proteins in the protoplasm would thereby be increased leading to greater swelling capacity. Such increase in *p*H was also found by Hoagland and Martin (1923) and Ballard (1933) using barley, and by

[3] Increase of water content with increasing nitrogen or protein content has been shown by other workers, e.g. Das (1936), and Garner et al (1934).

Pettinger (1931) using maize, although Böning and Böning-Seubert (1932) found the opposite effect in tobacco.

The relation between protein content and water content must be a complex one, since the water content itself is an important factor determining the protein content of leaves (Mothes, 1931; Petrie and Wood, 1938). A number of factors

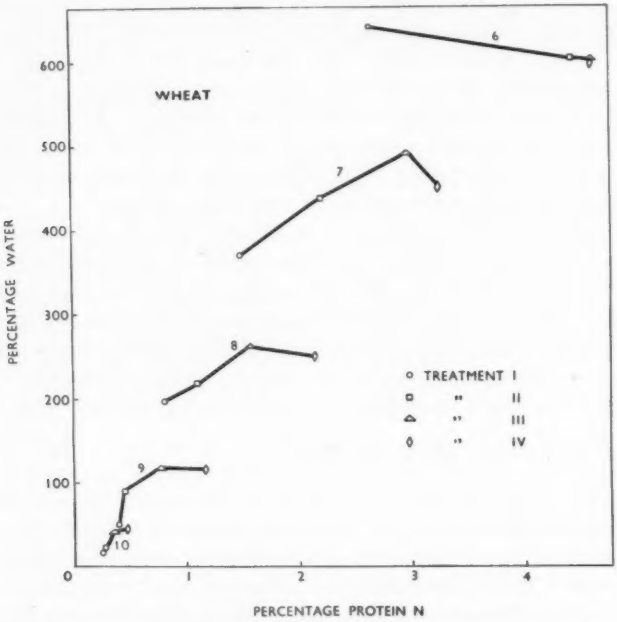


Fig. 6. Relation of water content to protein nitrogen content. The Arabic figures are harvest numbers. From data of Table 2.

must also contribute to the ontogenetic decrease in water content per unit of protein nitrogen. It is of interest to observe, however, that the relation is a characteristic one, and is similar for the two species considered.

SUMMARY.

This paper is a continuation of the account given in the first paper of the series of the effect of increasing over a certain range the initial nitrogen supply to wheat and Sudan grass. An account is now given of the effect of nitrogen supply on the drifting amounts in the leaves of protein nitrogen, soluble nitrogen, total phosphorus and water.

The absolute contents of these constituents of the total leaves rise to maxima

earlier than the maximum dry weight, and fall markedly during the latter part of the growing period. The absolute contents at first tend to be depressed by treatment, but later are increased, even in the case of phosphorus which was present in the same initial amounts in all treatments.

The percentage amounts, on a dry weight basis, have early maxima and decline throughout practically the whole growing period. Treatment effects are at first inappreciable, but later the contents of protein and soluble nitrogen and water become increased with treatment. In the case of wheat the phosphorus content becomes decreased with treatment, but is unaffected in Sudan grass.

Somewhat similar results are presented for the nitrogen and water contents of the third leaf in wheat.

It is suggested that the initial depression in dry weight produced by high nitrogen treatment is caused by a temporary reduction in water content of the plant, leading to reduced protein content of the leaves and a temporarily reduced unit leaf rate.

A synoptical account is given of the factors that determine the rates of import and export of nitrogen and phosphorus by the leaves. Possible factors in the present experiments are: (1) effect of external concentration; (2) rate of growth of leaves; (3) rate of growth of other parts and the nature of their development; (4) capacity of root system for intake; (5) rate of carbon assimilation; (6) internal changes in the leaves leading to decrease in net rate of protein synthesis; (7) establishment of a state of starvation with respect to some nutrient other than nitrogen.

The relation of the water content of the leaves to their protein nitrogen content is also discussed.

REFERENCES.

Ballard, L. A. T. (1933): Austral. J. exp. Biol., 11, p. 161.
Ballard, L. A. T. and Petrie, A. H. K. (1936): *Ibid.*, 14, p. 135.
Blanck, E. and Giesecke, F. (1934): J. f. Landw., 82, p. 33.
Böning, K. and Böning-Seubert, E. (1932): Biochem. Z., 247, p. 35.
Crist, J. W. (1926): Mich. State Coll. Agric. Exp. Sta. Tech. Bull. 74.
Crowther, F. (1934): Ann. Bot., 48, p. 877.
Das, U. K. (1936): Plant Physiol., 11, p. 251.
Davidson, J. (1933): J. Agric. Res., 46, p. 449.
Doneen, L. D. (1934): Wash. Agric. Exp. Sta. Bull. 296.
Garner, W. W., Bacon, C. W., Bowling, J. D. and Brown, D. E. (1934): U.S.A. Dept. Agric. Tech. Bull. No. 414.
Goedewaagen, M. A. J. (1929): Proc. Roy. Acad. Amsterdam, 32, p. 135.
Gregory, F. G. (1937): Ann. Rev. Biochem., 6, p. 557.
Hoagland, D. R. and Martin, J. C. (1923): Univ. of Calif. Tech. Paper No. 8.
McCalla, A. G. (1933): Canad. J. Res., 9, p. 542.
McCool, M. M. and Weldon, M. D. (1928): J. Amer. Soc. Agron., 20, p. 778.
Mason, T. G. and Maskell, E. J. (1931): Ann. Bot., 45, p. 125.
Mason, T. G. and Maskell, E. J. (1934): *Ibid.*, 48, p. 119.
Mevius, W. (1928): Z. f. Pflanzenernähr., A, 10, p. 208.

Mevius, W. and Engel, H. (1929): Planta, 9, p. 1.
Mothes, K. (1926): Planta, 1, p. 472.
Mothes, K. (1931): Planta, 12, p. 686.
Needham, J. (1933): Biol. Revs., 8, p. 180.
Nightingale, G. T. (1934): Bot. Gaz., 95, p. 437.
Pearsall, W. H. and Ewing, J. (1929): Ann. Bot., 43, p. 27.
Petrie, A. H. K. and Wood, J. G. (1938): Ann. Bot., N.S., 2 (in press).
Pettinger, N. A. (1931): J. Agric. Res., 49, p. 95.
Rippel, A., Behr, G. and Meyer, R. (1933): Z. f. Pflanzenernähr., A, 32, p. 95.
Rippel, A. and Meyer, R. (1933): *Ibid.*, A, 27, p. 257.
Sekera, F. (1928): *Ibid.*, B, 7, p. 533.
Smirnow, A. I. (1928): Planta, 6, p. 687.
Tiedjens, V. A. (1934): Plant Physiol., 9, p. 31.
Thomas, W. (1927): Science, 66, p. 115.
Williams, R. F. (1936): Austral. J. exp. Biol., 14, p. 165.
Willis, L. G. and Davis, E. A. (1928): North Carolina Agric. Exp. Sta. Tech. Bull. No. 30.
Zinzadzé, C. (1931): Proc. Internat. Soc. Soil Sci., 6, p. 95.

ERRATA IN PREVIOUS PAPERS OF THIS SERIES.

1. Ballard and Petrie (1936).

   p. 146, line 17. For "S.E. for means of 6" *read* "S.E. for means of 3".
   p. 146, penultimate line. For "S.E. for means of 6" *read* "S.E. for means of 5".
   p. 146, last line. For "S.E. for means of 5" *read* "S.E. for means of 3".

2. Williams (1936).

   p. 181, line 30. For "0·0138" *read* "0·138".

SOME EFFECTS OF ORGANIC SOLVENTS ON THE BLOOD PIGMENT

by

H. F. HOLDEN AND C. G. SETTER

(From the Walter and Eliza Hall Institute, Melbourne).

(Submitted for publication 27th July, 1937.)

It has long been known that such substances as ethyl alcohol or acetone, when added in large amount to a solution of oxyhaemoglobin precipitate the pigment in the form of a reddish mass, which is insoluble in water, and whose spectrum shows two very ill-defined bands in the yellow-green portion. Such substances come within the category of "parahaematins". On solution in alkali there is obtained alkaline haematin, mixed with a certain amount of alkaline methaemoglobin. The researches described below are undertaken with a view to seeing whether any factors would modify to any great extent the amount of native protein recoverable from the solid. In a previous paper (Holden, 1937) it was shown that if reduced haemoglobin were precipitated by ethyl alcohol in the absence of oxygen at room temperature, very little "irrenaturable" protein was formed. From this and other experiments described in that paper the inference was drawn that the denaturation of oxyhaemoglobin by organic solvents was due, in part at least, to an oxidation of the globin coupled to that of the reduced haematin when the specific bond between them was loosened by the action of the solvent.

The first group of experiments described in this paper was conducted as follows: Solutions of oxyhaemoglobin of a number of different species were prepared and the stroma removed by adjustment of the reaction and centrifugation. The oxyhaemoglobin of a portion of each specimen was precipitated by alcohol at laboratory temperature, 18°–22° C., the precipitate washed with absolute alcohol, and dried in a vacuum desiccator. The remainder was precipitated with acetone below 0° C., the precipitate washed with dry acetone and similarly dried. With a view to obtaining comparative values for the amount of native protein recoverable on solution, small samples were dissolved with the help of the least amount of sodium hydroxide or acetic acid which was found capable of effecting solution. The solution was suitably diluted and adjusted to a suitable hydrogen ion concentration for the formation of methaemoglobin. Its pH was determined with the glass electrode. The extinction coefficient Σ of the peak of the Soret band was measured with a Hilger ultra-violet spectrophotometer. The results, which appear

in Table 1, are the means of several determinations of which the experimental error is about 5 p.c. of the value.

TABLE 1.

Species.	Denaturant.	Solvent.	C.c. of N/10 solvent per mg. solid, required.	P.c. Native protein recovered.
Cat	C_2H_5OH	NaOH	0·02	42
	$(CH_3)_2CO$	NaOH	0·02	45
	C_2H_5OH	CH_3COOH	0·01	56
	$(CH_3)_2CO$	CH_3COOH	0·01	53
	$(CH_3)_2CO$	NH_3	0·016	52
Guinea-pig	C_2H_5OH	NaOH	0·01	56
	$(CH_3)_2CO$	NaOH	0·02	47
	C_2H_5OH	CH_3COOH	0·04	51
	$(CH_3)_2CO$	CH_3COOH	0·045	47
Horse	C_2H_5OH	NaOH	0·01	53
	$(CH_3)_2CO$	NaOH	0·02	51
	C_2H_5OH	CH_3COOH	0·02	51
	$(CH_3)_2CO$	CH_3COOH	0·02	51
Man	C_2H_5OH	NaOH	0·01	51
	$(CH_3)_2CO$	NaOH	0·02	47
	C_2H_5OH	CH_3COOH	0·012	49
	$(CH_3)_2CO$	CH_3COOH	0·012	47
Pig	C_2H_5OH	NaOH	0·01	52
	$(CH_3)_2CO$	NaOH	0·02	40
	C_2H_5OH	CH_3COOH	0·012	46
	$(CH_3)_2CO$	CH_3COOH	insoluble	—
Rabbit	C_2H_5OH	NaOH	0·02	47
	$(CH_3)_2CO$	NaOH	0·02	42
	C_2H_5OH	CH_3COOH	0·014	45
	$(CH_3)_2CO$	CH_3COOH	0·013	48
Sheep	C_2H_5OH	NaOH	0·02	49
	$(CH_3)_2CO$	NaOH	0·02	49
	C_2H_5OH	CH_3COOH	0·022	40
	$(CH_3)_2CO$	CH_3COOH	0·02	53

The differences obtained are not, in general, very striking, but certain points may be noted:

The highest proportion of methaemoglobin was obtained from those which were most readily soluble. Acetone at 0° C. was only a little less destructive than alcohol at laboratory temperature. Usually the precipitate would not completely dissolve in dilute ammonia, but in one case where this was accomplished more methaemoglobin was recovered than when the same specimen was dissolved in dilute sodium hydroxide. On solution in alkali the spectrum seen immediately was that of a mixture of alkaline haematin and alkaline methaemoglobin. In solution in acid, on the other hand, the immediate spectrum was that of a parahaematin only. The methaemoglobin band was invisible and only slowly developed on standing. It was necessary to wait one–two hours to obtain the maximum Σ. Thus renaturation both in acid and in alkaline solution was to some extent unavoidably complicated by the irreversible denaturation which (especially in alkali) occurs under such circumstances. To this fact are attributable the slightly lower

figures usually obtained when the precipitates were dissolved with sodium hydroxide. The oxyhaemoglobin most resistant to irreversible denaturation appeared to be that of the cat, closely followed by that of the horse, while that of the rabbit was most damaged. That of the pig was much affected by acetone, the precipitate being insoluble in dilute acetic acid.

Some cat oxyhaemoglobin was precipitated by alcohol at room temperature at pHs 6·2, 7·3, 7·6, but there was no difference in the results. Similar results were also obtained when some cat erythrocytes were haemolysed with saponin and to the resultant solution containing the stroma was added an excess of alcohol. Some experiments were also done on the effect of certain other colloids on the precipitation of rabbit oxyhaemoglobin by alcohol. A weight of ox serum albumin equal to that of the oxyhaemoglobin present was without effect, as was an amount of rabbit serum containing a weight of mixed proteins equal to that of the oxyhaemoglobin.

When a weight of lysalbic acid, prepared from egg white, equal to the weight of oxyhaemoglobin was present the pigment could be precipitated by alcohol and redissolved with most of it still in the form of oxyhaemoglobin. An equal weight of gum ghatti was even better, and even when only 1 gm. of gum ghatti was present for each 4 gm. of oxyhaemoglobin only some 20 p.c. was denatured, the remainder staying as oxyhaemoglobin. In these experiments with protective colloids the precipitates were not dried, but redissolved after filtering off the alcohol by suction.

Some rabbit oxyhaemoglobin solution was placed in the tonometer described in a previous paper (Holden, 1937) and after being equilibrated three times with pure hydrogen was treated, still in the tonometer with oxygen-free acetone containing HCl, at laboratory temperature. The protein was nearly all irrenaturable but was of a pure white colour, in contrast to preparations of irrenaturable globin made in the presence of oxygen which always have a faint yellowish tinge due to pigment which is insoluble in acetone or alcohol and ether. It would now appear that this pigment is an artifact. The same experiment was carried out at 0° C., in the hope that the yield of native globin recovered would be materially increased. This was however found not to be the case. On the other hand the resultant globin solution was remarkably free from traces of pigment and was a very attractive reagent for spectroscopic studies.

DISCUSSION.

Although the experiments in this paper throw little further light on the mechanism of the denaturation of the blood pigments, they offer one or two suggestions: There would appear to be two kinds of bond between each molecule of the pigment and the molecule of native globin. One is similar to that between reduced haematin and the tertiary nitrogen of denatured globin, other proteins or other simple nitrogenous substances, such as pyridine or histidine, and gives rise to the classes of substances known as parahaematins and haemochromogens respectively.

The other bond is peculiar to native globin and is at present of an unknown character. Native globin parahaematin and haemochromogen (if preparable) revert too rapidly to methaemoglobin and haemoglobin respectively, for their existence to be directly demonstrable. It must, however, be remembered that the parahaematin prepared by Anson and Mirsky (1934) and the haemochromogen prepared by Holden (1937) by the action of sodium salicylate on methaemoglobin and haemoglobin respectively are not in themselves evidence of the denaturation of these compounds since no precipitate was formed, unless of course spectroscopic evidence is made the definition of denaturation. A reversible loosening of the specific globin bond while retaining the nonspecific ones would (at present) meet this case equally well. Since the Fe″ atom is engaged in the formation of haemochromogens it is assumed that the Fe‴ atom is similarly concerned in the formation of parahaematins. Evidence is accumulating in this laboratory that the latter assumption may not be justified, and that links between pigments and protein are possible which do not involve a metallic atom. The effect of gum ghatti on the denaturation is peculiar and at present does not admit of any explanation.

SUMMARY.

The amount of renaturation possible after denaturation by alcohol and by acetone of oxyhaemoglobin of different species has been determined. The presence of gum ghatti or lysalbic acid greatly increased the amount. That of serum proteins was without effect.

ACKNOWLEDGMENT.

The authors are indebted to Professor W. A. Osborne, Professor of Physiology in the University of Melbourne, for his generous loan of a spectrophotometer.

REFERENCES.

Anson, M. L. and Mirsky, A. E.: J. gen. Physiol., 17, p. 399.
Holden, H. F. (1937): Austral. J. exp. Biol., 15, p. 43.

THE COMBINATION WITH PROTEINS OF CERTAIN PORPHYRINS

by

HENRY FRANCIS HOLDEN

(From the Walter and Eliza Hall Institute, Melbourne).

(Submitted for publication 7th October, 1937.)

Hill and Holden (1926) observed changes in the visible absorption spectrum of protoporphyrin in the presence of globin which they interpreted as an indication of the formation of a compound between these substances. Haurowitz and Waelsch (1929) showed that similar spectral changes occurred when horse serum was added to a solution of haematoporphyrin in dilute alkali. They attributed the effects to adsorption of the porphyrin by the serum protein and the subject has since remained in this state. The correct interpretation bears on the constitution of the blood pigment, since a knowledge of the nature of the active portions of the globin molecule which can combine with reduced haematin to yield haemoglobin involves some knowledge of the precise groups of the haematin molecule with which they react. The questions remain whether a metallic element is essential for a porphyrin to combine with globin, and whether there is anything specific in the nature of globin in its reactions with porphyrins as there is in its reactions with haematin. Haurowitz and Waelsch (1929) believed that only iron-porphyrins (haematins) could form definite compounds with globin. They regarded the effect of globin and of horse serum on the spectrum of haematoporphyrin as due to adsorption.

The researches to be described in this paper suggest that the answer to the first question needs revision, that porphyrins do combine with proteins in the absence of a metal, but that, as Haurowitz stated, for globin to show its specific effects a metal is necessary. Whether iron is the only metal to effect this specific combination is as yet uncertain.

The work described below consists chiefly of spectrophotometric measurements in the ultra-violet region of three substances, haemin, protoporphyrin and haematoporphyrin under different hydrogen ion concentrations and in the presence of various proteins and other colloids. The apparatus and general procedure is described in a previous paper (Holden, 1936).

The hypothesis adopted for the discussion of the results of the experiments is that the intense narrow band at about 4000Å. is due to that proportion of the pigment which is in separate molecules, either in simple solution or held on the surface of a large molecule of a colloid outside each other's sphere of influence. The broad,

less defined band represents the portion which is aggregated and in colloidal solution whether protected by a lyophile colloid or not.

The haematoporphyrin was kindly given by Mr. R. Hill; the haemin and protoporphyrin were prepared in this laboratory, the latter by the method of Hill and Holden (1926).

TABLE 1.

Haematin.

Plate.	pH.	$\epsilon 10^{-3}$ maximum	$\nu 10^{-12}$	Solvent.	Substance added.
148	—	136	753	C_2H_5OH	HCl
207	—	56	725	C_5H_5N	—
216	7·1	43	789	H_2O	gum ghatti
218	7·2	46	736	H_2O	horse serum
170	4·9	153	737	H_2O	rabbit globin
273	2·8	43	803	H_2O	
274	2·9	45	803	H_2O	
281	5·3	84	736	H_2O	denatured
277	6·2	85	737	H_2O	rabbit globin
278	6·9	84	736	H_2O	
280	7·7	64	736	H_2O	
279	8·2	61	737	H_2O	
302	9·6	78	731	H_2O	denatured
303	4·3	115	737	H_2O	ox globin

NOTICE.

1. The small effect on ϵ of horse serum compared with globin.
2. The constancy of ϵ between pH 5·3–6·9 for denatured rabbit globin suggests the presence of native globin, since the decrease of ϵ of methaemoglobin would compensate for an increase in ϵ of parahaematin.
3. Plate 303 was taken on a solution previously set to pH 9·6, and afterwards acidified before photography. This is the surest test for methaemoglobin, implying the presence in the denatured ox globin of native globin (or renaturable globin).
4. Graph No. 1 has curves for neutral haematin, for the effect of horse serum, and of native globin.

If the sodium salt of protoporphyrin was dissolved in absolute alcohol containing a little hydrochloric acid and immediately reprecipitated with sodium hydroxide a small amount of impurity with a low absorption in the region of 4000Å. was eliminated. This step was accordingly taken. Most of the experiments on haematoporphyrin in acid solution were done on solutions diluted from an alkaline solution of haematoporphyrin, with the addition of suitable amounts of hydrochloric acid and of a buffer or protective colloid when necessary. A few experiments were done in this way on protoporphyrin. The other solutions were made by adding a small volume of a solution of the porphyrin or haematin in alcoholic hydrochloric acid to the other constituents of the final mixture in a graduated flask. Sufficient details are given in the appropriate section to enable similar solutions to be prepared. Control experiments showed that the two methods gave similar results. Haematin undergoes some change in alkaline solutions fairly rapidly which renders their use unsuitable for quantitative work.

TABLE 2.

Haematoporphyrin.

Plate.	pH.	$\epsilon 10^{-3}$ maximum	$\nu 10^{-12}$	Solvent.	Substance added.
24	—	266	726	C_2H_5OH	HCl
213	—	145	737	C_5H_5N	—
102	1·1	357	748	H_2O	HCl
79	2·4	326	747	H_2O	HCl
80	2·7	300	750	H_2O	HCl
76	3·0	274	748	H_2O	HCl
99	3·1	203	748	H_2O	HCl
81	3·7	90	798	H_2O	HCl
70	4·2	94	798	H_2O	HCl
85	4·5	82	801	H_2O	HCl
84	4·8	82	796	H_2O	HCl
68	5·7	94	793	H_2O	HCl
52	6·8	107	763	H_2O	NaOH
66	6·9	110	765	H_2O	HCl
96	7·1	122	763	H_2O	—
65	7·3	130	762	H_2O	—
63	7·7	141	764	H_2O	—
55	8·2	135	762	H_2O	—
57	9·0	135	760	H_2O	NaOH
61	11	129	761	H_2O	NaOH
89	4·8	81	787	H_2O	Horse serum
87	7·1	135	763	H_2O	Horse serum
94	4·2	76	796	H_2O	
95	6·9	90	788	H_2O	protalbic acid
92	7·8	128	756	H_2O	
251	5·1	89	753	H_2O	
260	5·2	94	756	H_2O	denatured
258	6·4	125	753	H_2O	rabbit globin
257	7·9	137	756	H_2O	
256	8·4	130	757	H_2O	
248	4·3	76	754	H_2O	
246	5·4	117	750	H_2O	
242	5·6	141	753	H_2O	rabbit globin
142	6·6	164	751	H_2O	
244	6·6	163	753	H_2O	

NOTICE.

1. The small effect of horse serum.
2. The ν in the presence of rabbit globin is nearer to that in acid solution than to that in alkaline solution.
3. Plate 52 was made from a solution in alcoholic HCl, the others of the free porphyrin plates from solutions in dilute NaOH.
4. Protalbic acid, though an excellent protective colloid has no effect.
5. Graph No. 2 has curves for haematoporphyrin alone, in the presence of globin, of horse serum and for alkaline haematoporphyrin.

The sodium salt of protoporphyrin dissolves in water in the form of colloidal aggregates. The solution, unlike that of haematoporphyrin, contains so few free single molecules of the porphyrin that its spectrum, unlike that of alkaline haematoporphyrin, is unchanged by the addition of globin or of serum. The whole question of the relation of the degree of dispersion of a pigment to its absorption spectrum is dealt with by Keilin (1926). Fresh solutions of the pigments, proteins, etc., were prepared for each day's work. The pyridine was dried over

sodium hydroxide and finally distilled. The hydrazine was similarly dried. The gum ghatti was purified by solution in water, centrifuging away insoluble matter and precipitation by alcohol. The globin was prepared by the precipitation at 0° C. of acid haematin with acetone in an atmosphere of pure hydrogen. The avoidance of oxidation reduces the colour of the final product. A study was made of the ultra-violet absorption band of haemato- and protoporphyrins in aqueous solution at different concentrations of hydrogen ions. Next a study was made at similar hydrogen ion concentrations when the porphyrin solution was added to aqueous solutions of proteins or other colloids. A glass electrode was used for the pH determinations.

TABLE 3.

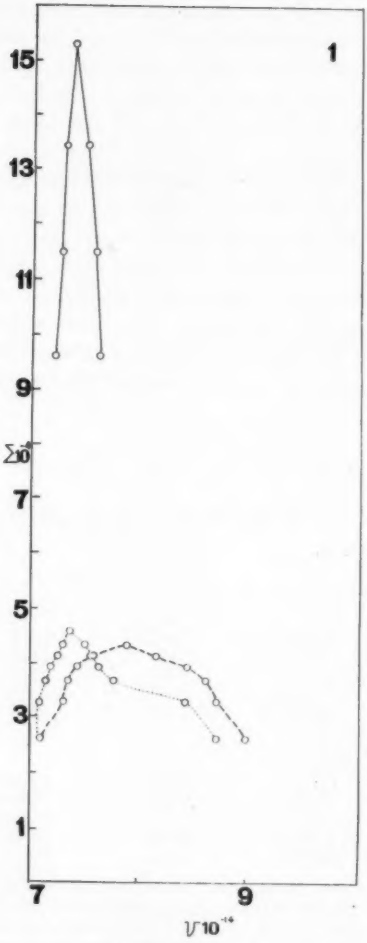
Protoporphyrin.

Plate.	pH.	$\epsilon 10^{-3}$ maximum	$\nu 10^{-12}$	Solvent.	Substance added.
34	—	183	719	C_2H_5OH	HCl
205	—	96	725	C_5H_5N	—
116	0·1	171	737	H_2O	HCl
109	1·0	129	737	H_2O	HCl
121	1·3	106	735	H_2O	HCl
122	1·9	35	813	H_2O	HCl
284	3·1	31	830	H_2O	HCl, gum ghatti, sodium citrate
283	3·8	33	838	H_2O	
286	5·3	33	782	H_2O	
40	7·6	38	843	H_2O	
124	2·9	34	736	H_2O	horse serum
192	3·9	40	738	H_2O	
194	7·4	61	737	H_2O	
191	6·6	35	826	H_2O	casein
189	7·7	40	782	H_2O	
125	6·6	42	784	H_2O	protalbic acid
289	6·7	40	789	H_2O	
288	7·5	49	814	H_2O	
291	8·8	42	787	H_2O	
264	5·0	60	737	H_2O	denatured rabbit globin
265	5·6	66	739	H_2O	
266	6·9	70	737	H_2O	
268	7·9	74	737	H_2O	
270	8·7	44	737	H_2O	
233	6·6	82	737	H_2O	rabbit globin
230	7·1	113	736	H_2O	
235	7·6	108	736	H_2O	
237	7·9	98	736	H_2O	
238	8·4	81	735	H_2O	

NOTICE.

1. The vague outline of the curve for free protoporphyrin renders the value for the ν for its maximum quite uncertain.
2. The effect of horse serum though discernible is small.
3. Graph No. 3 has curves for the free porphyrin, for the effects of horse serum and of native globin.

Some similar experiments were performed with haematin. The pigments were also examined when dissolved in pyridine. Some measurements of the wavelengths of the visible bands were carried out with the Hartridge reversion spectro-



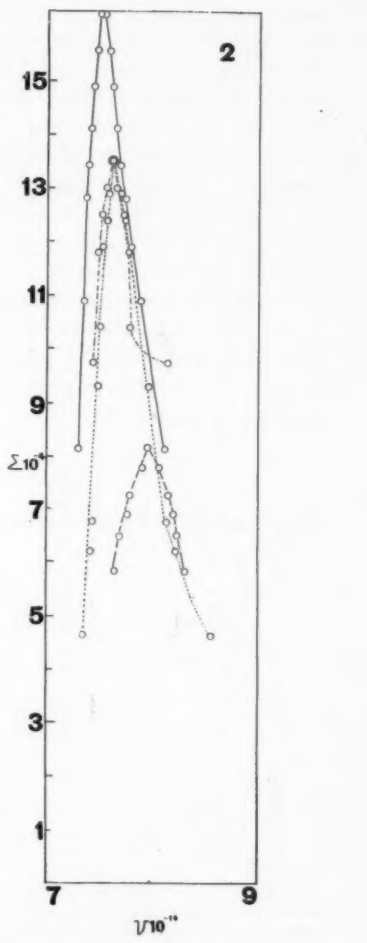
Graph No. 1.

Abscissae = $\nu 10^{-14}$
Ordinates = $\epsilon 10^{-4}$

$$\epsilon = \log \frac{I_0}{I} \frac{M}{cd}$$

where M = Molecular weight.
I_0 = Intensity of entering beam.
I = Intensity of emergent beam.
c = No. of grams in one litre.
d = Depth of liquid in cm.
ν = Frequency.

— — — = Haematin (neutral).
———— = Haematin and globin (methaemoglobin).
– – – – – = Haematin and horse serum.



Graph No. 2.

Abscissae = $\nu 10^{-14}$
Ordinates = $\epsilon 10^{-4}$

$$\epsilon = \log \frac{I_0}{I} \frac{M}{cd}$$

where M = Molecular weight.
I_0 = Intensity of entering beam.
I = Intensity of emergent beam.
c = No. of grams in one litre.
d = Depth of liquid in cm.
ν = Frequency.

— — — = Haematoporphyrin.
– – – – – = Haematoporphyrin (alkaline).
———— = Haematoporphyrin and globin.
– – – – = Haematoporphyrin and horse serum.

meter (see Table 4). In neutral solution, a high concentration of horse serum sharpens the visible bands of protoporphyrin and shifts them towards the violet end of the spectrum as was observed in the case of haematoporphyrin by Haurowitz and Waelsch (1929). The porphyrin must however be added dissolved in alcoholic hydrochloric acid. The spectrum of haematoporphyrin dissolved in alkali differs from that of the free porphyrin protected by gum ghatti at pH 4·5. Carefully purified protoporphyrin, dissolved as its sodium salt in distilled water, has a somewhat different visible spectrum from the same substance in solution in the presence of small amounts of salts, which appear to have an aggregating effect on the pigment molecules. Pyridine and hydrazine dissolved both porphyrins with a decrease in the wave length and increase in sharpness of the bands, compared with those of the free porphyrins dissolved in water. The effect was analogous to that of globin already described by Hill and Holden (1926). The porphyrins dissolved in piperidine had the same spectra as those of the free porphyrins in water.

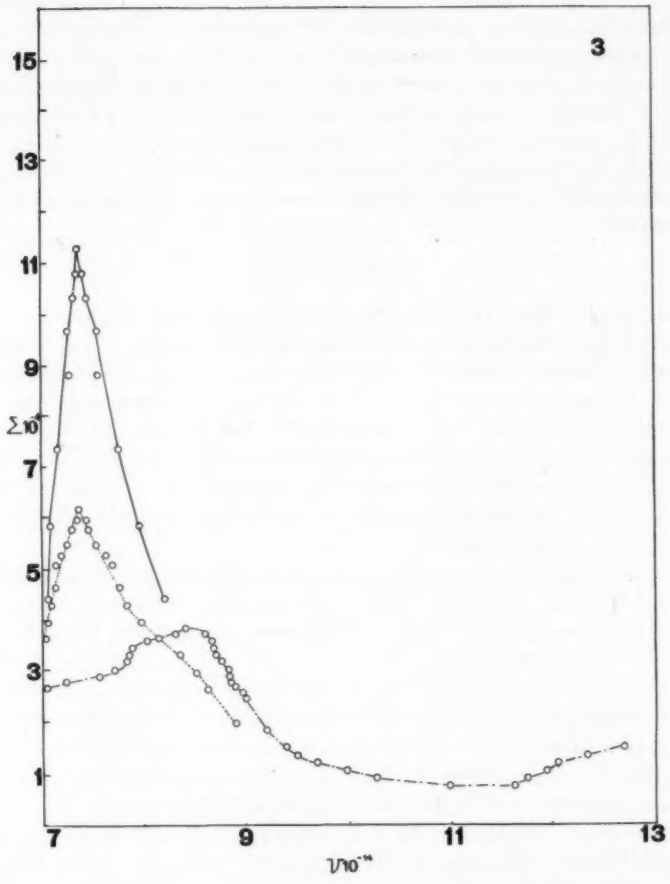
TABLE 4.

Wavelengths of Bands in the Visible Spectrum as Determined by the Hartridge Spectrometer.

Solvent.	α	β	γ	δ
HAEMATOPORPHYRIN.				
NaOH	6,105	5,719	5,407	5,053
H_2O and gum ghatti pH 4·5	6,251	5,730	5,376	5,039
piperidine	6,250	5,755	5,340	4,975
hydrazine	6,184	5.658	5,347	4,902
pyridine	6,225	5,677	5,312	4,983
PROTOPORPHYRIN.				
H_2O	6,428	5,927	5,398	obscure
H_2O and salt	6,438	5,929	5,390	obscure
horse serum	6,332	5,765	5,407	obscure
pyridine	6,312	5,758	5,410	5,000
hydrazine	6,371	5,815	5,433	5,096

The solutions in horse serum and in hydrazine were prepared by adding a little of a strong solution of protoporphyrin in alcoholic HCl to an alkaline solution of horse serum and to hydrazine respectively. An analogous method was used for making the solution of haematoporphyrin in piperidine, but the spectrum was that of the free porphyrin. Piperidine was also without much effect on the spectrum of protoporphyrin.

Attempts were made to alter the position of the visible bands of the porphyrin spectra by adding their acid solutions to concentrated solutions of nitrogenous substances, containing sufficient alkali to neutralize the acid. No effect, however, was observed. The substances tried included histidine, α, α, di-pyridyl (in alcohol), iminazole, 4 methyl iminazole and potassium cyanide. These substances all combine with reduced haematin in alkaline solution and some form parahaematins, though highly dissociated ones. No protein is known which gives so little dissociated a parahaematin as denatured globin. When, however, an attempt was made



Graph No. 3.

Abscissae = $\nu 10^{-14}$
Ordinates = $\epsilon 10^{-4}$

$$\epsilon = \log \frac{I_0}{I} \frac{M}{cd}$$

where M = Molecular weight.
I_0 = Intensity of entering beam.
I = Intensity of emergent beam.
c = No. of grams in one litre.
d = Depth of liquid in cm.
ν = Frequency.

– – – – – – – = Protoporphyrin.
——————— = Protoporphyrin and globin.
– – – – – – – = Protoporphyrin and horse serum.

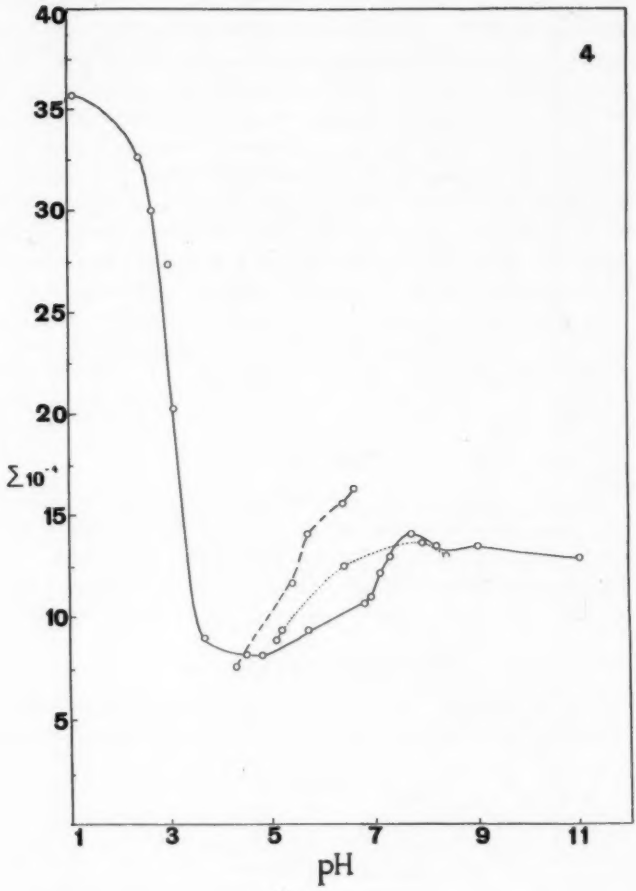
to study compounds of porphyrins with denatured globin an unexpected obstacle was present which renders uncertain the interpretation of all the results obtained with this protein. All the specimens prepared (ox, horse and rabbit) were found to contain some renaturable or native globin which could not be separated from the irrenaturable globin. Various methods were tried for obtaining a pure irrenaturable globin but without success. The effect observed with the specimens used is such as would be expected on the basis of the amount of native protein found on treating the specimen with haematin and measuring the Σ of the absorption band of the product.

DISCUSSION.

It can be seen from the data that haematin and protoporphyrin can form compounds with a protein in horse serum and with native rabbit globin. The results with casein and protalbic acid do not indicate any such compound. The figures for haematoporphyrin do not show any special compound except in the case of globin. It must however be remembered that the case of this porphyrin is rendered more difficult of interpretation by the fact that so much of it appears to exist in solution in the form of free molecules which are not, as in the case of protoporphyrin, readily caused to aggregate by such simple means as the addition of small quantities of electrolytes.

Again in the concentrations that had to be used globin appears to be unstable in even faintly alkaline solution, rendering it uncertain whether the slope of that portion of the protoporphyrin-globin curve between pH 7 and pH 8·4 is due to dissociation or denaturation (see Table 3).

The observations in this paper are in accordance with the idea that these porphyrin compounds are analogous to parahaematins since they appear to be least dissociated in neutral solution. While it is true that haemochromogens in suitable concentration on neutralization and oxidation yield parahaematins it has not been proved that the iron atom in the parahaematins plays the part that it does in haemochromogens. The ultra-violet absorption curve of alkaline methaemoglobin (Hicks and Holden, 1929) suggests that it is either a mixture of alkaline haematin and methaemoglobin or a relatively little dissociated (native) globin parahaematin. Its visible spectrum accords better with the latter hypothesis, as does the fact that on adjusting its solution to pH 4·5 the ultra-violet methaemoglobin band regains its original intensity, showing that no irrenaturable globin had been formed. Whether the parahaematin formed temporarily in the course of renaturation when an acidified solution of methaemoglobin containing acid haematin and denatured globin is brought to pH 6 is globin parahaematin or renaturable globin parahaematin is still not certain. Its visible spectrum is much less clear than that of alkaline methaemoglobin. Oxyhaemoglobin, carbon monoxide haemoglobin and reduced haemoglobin are more stable to many chemical reagents than methaemoglobin. Thus methaemoglobin is converted completely to acid haematin at pH 3 while the other pigments have to reach pH 2 before they are decomposed. Methae-



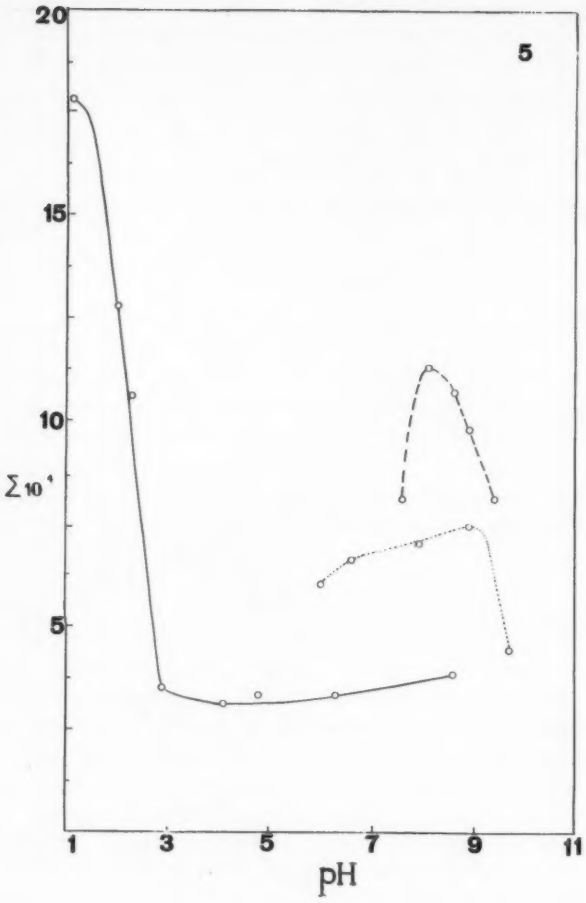
Graph No. 4.

Abscissae = pH.
Ordinates = $\epsilon 10^{-4}$ of the maximum.

$$\epsilon = \log \frac{I_0}{I} \frac{M}{cd}$$

where M = Molecular weight.
I_0 = Intensity of entering beam.
I = Intensity of emergent beam.
c = No. of grams in one litre.
d = Depth of liquid in cm.

———— = Haematoporphyrin.
— — — — — = Haematoporphyrin and globin.
- - - - - - - - = Haematoporphyrin and "denatured" globin.



Graph No. 5.

Abscissae = pH.
Ordinates = $\epsilon 10^{-4}$ of the maximum.

$$\epsilon = \log \frac{I_0}{I} \frac{M}{cd}$$

where M = Molecular weight.
I_0 = Intensity of entering beam.
I = Intensity of emergent beam.
c = No. of grams in one litre.
d = Depth of liquid in cm.

———— = Protoporphyrin.
— — — — — = Protoporphyrin and globin.
- - - - - - - - - = Protoporphyrin and "denatured" globin.

moglobin is converted to parahaematin by a concentration of sodium salicylate which has no effect on oxy- or reduced haemoglobin. Protoporphyrin globin can only exist near the neutral point. Reduced haematin readily combines with many proteins to form haemochromogens in strongly alkaline solution while haematin can apparently only combine with a few proteins in neutral solution. With simpler nitrogenous compounds the difference is equally marked. A trace of pyridine converts reduced haematin to pyridine haemochromogen but pyridine parahaematin is formed only in very high concentrations of pyridine. The same is true of piperidine, iminazole and hydrazine. This difference in stability runs parallel with the presence or absence of a reduced iron atom. From the graphs it can be seen that the relative effects of globin and horse serum on the ultra-violet absorption spectrum of protoporphyrin are, in neutral solution, comparable with their relative effects on that of haematin. Methaemoglobin is judged to be a compound and the spectrometric evidence favours the idea of a somewhat similar structure for protoporphyrin globin.

SUMMARY.

In neutral solution globin changes the u-v absorption spectra of protoporphyrin and of haematoporphyrin. Similar but smaller changes are effected by horse serum. Casein and gum ghatti have no effect. It is suggested that the differences observed indicate the formation of porphyrin-protein compounds analogous to haematin protein compounds (parahaematins).

ACKNOWLEDGMENT.

The writer is indebted to Professor W. A. Osborne of the University of Melbourne for the generous loan of an ultra-violet spectro-photometer.

REFERENCES.

Haurowitz, F. and Waelsch, H. (1929): Z. physiol. Chem., 182, p. 82.
Hicks, C. S. and Holden, H. F. (1929): Austral. J. exp. Biol., 6, p. 175.
Hill, R. and Holden, H. F. (1926): Biochem. J., 20, p. 1326.
Holden, H. F. (1936): Austral. J. exp. Biol., 14, p. 291.
Keilin, D. (1926): Proc. Roy. Soc., London, 100 B, p. 129.

APPENDIX.

DETAILS OF THE COMPOSITION OF THE SOLUTIONS FOR PHOTOGRAPHY.

Throughout this section the following abbreviations are employed:

S = Stock solution of the pigment.
Hph 2 HCl = haematoporphyrin bi-hydrochloride.
Pph Na = sodium salt of protoporphyrin.
d.r.g. = denatured rabbit globin. 5 mg. per c.c. in 0·01 N.HCl.
d.o.g. = denatured ox globin. 5 mg. per c.c. in 0·01 N.HCl.
g.g. = gum ghatti solution 1 p.c.

Unless otherwise stated the NaOH and HCl were 0·1 N solutions. The solutions were placed in standard flasks in the order named, and the last-named run in to the mark. The NH_3 was about

0·1 N. Its exact strength is unimportant as slight differences in the different protein preparations alter the amount necessary which must be determined by a preliminary titration to the desired pH.

The composition of the standard buffer solutions used may conveniently be found in W. M. Clark's "The Determination of Hydrogen Ions".

HAEMATIN.

Plate	
148	1·28 mg. haemin in 0·2 c.c. NaOH, 0·1 N alcoholic HCl to 10 c.c. = S. 0·3 c.c. S, 0·1 N alcoholic HCl to 10 c.c.
170	1·70 mg. haemin in 0·2 c.c. NaOH, 9 c.c. absolute alcohol, 0·1 c.c. 10 N.HCl, alcohol to 10 c.c. = S. 0·6 c.c. of 0·5 p.c. rabbit globin, 0·2 c.c. NH_3, 5 c.c. H_2O, 0·2 c.c. S, H_2O to 10 c.c.
207	3·51 mg. haemin in 10 c.c. pyridine = S. 0·2 c.c. S, pyridine to 10 c.c.
216–218	1·99 mg. haemin dissolved as for plate 170 = S.
216	0·5 c.c. g.g., 5 c.c. H_2O, 0·5 c.c. S, 0·5 c.c. Clark's pH 7·6 phosphate buffer. 0·5 c.c. NaOH, H_2O to 10 c.c.
218	as 216 but 0·1 c.c. horse serum instead of g.g.
273–281	1·84 mg. haemin dissolved as for plate 170 = S.
273	1 c.c. d.r.g., 0·5 c.c. NH_3, 0·5 c.c. S, H_2O to 10 c.c.
274	as 273 but 0·54 c.c. NH_3.
277	1 c.c. d.r.g., 0·8 c.c. NH_3, 1 c.c. g.g., 0·33 c.c. S, H_2O to 10 c.c.
278	0·7 c.c. d.r.g., 0·7 c.c. g.g., 0·7 c.c. 1·8 p.c. NaCl, 0·6 c.c. NH_3, 0·35 c.c. S, H_2O to 10 c.c
279	as 278 but 0·70 c.c. NH_3.
280	as 278 but 0·65 c.c. NH_3.
281	as 278 but 0·56 c.c. NH_3.
302–303	2·08 mg. haemin in 0·25 c.c. NaOH, 9 c.c. absolute alcohol, 0·08 c.c. 10 N. HCl, alcohol to 10 c.c. = S.
302	0·5 c.c. g.g., 0·5 c.c. 1·8 p.c. NaCl, 0·6 c.c. NH_3, 1 c.c. d.o.g., 0·3 c.c. NaOH, 5 c.c. H_2O, 0·3 c.c. S, H_2O to 10 c.c.
303	1 c.c. d.o.g., 0·5 c.c. NaOH, 0·3 c.c. S, 5 c.c. H_2O, 0·15 c.c. HCl, H_2O to 10 c.c.

HAEMATOPORPHYRIN.

99–102	2·46 mg. Hph 2 HCl in 0·5 c.c. NaOH, H_2O to 10 c.c. = S.
99	0·7 c.c. S, 50 c.c. H_2O, 1 c.c. HCl, H_2O to 100 c.c.
102	0·6 c.c. S, HCl to 100 c.c.
76–81	4·39 mg. Hph 2 HCl in 0·8 c.c. NaOH, H_2O to 10 c.c. = S.
76	1 c.c. S, 100 c.c. H_2O, 5 c.c. HCl, H_2O to 250 c.c.
79	as 76 but 10 c.c. HCl.
80	as 76 but 7 c.c. HCl.
81	1 c.c. S, 50 c.c. H_2O, 0·4 c.c. HCl, H_2O to 100 c.c.
63–70	2·10 mg. Hph 2 HCl in 0·4 c.c. NaOH, H_2O to 10 c.c. = S.
63	1 c.c. S, H_2O to 50 c.c.
65	1 c.c. S, 0·3 c.c. M. NaCl, H_2O to 50 c.c.
66	1 c.c. S, 0·2 c.c. HCl, H_2O to 50 c.c.
68	1 c.c. S, 0·5 c.c. HCl, H_2O to 50 c.c.
70	1 c.c. S, 1 c.c. HCl, H_2O to 50 c.c.
84–89	2·03 mg. Hph 2 HCl in 0·4 c.c. NaOH, H_2O to 10 c.c. = S.
84	2·5 c.c. S, 50 c.c. H_2O, 1·4 c.c. HCl, H_2O to 100 c.c.
85	as 84 but 1·8 c.c. HCl.
87	1 c.c. S, 0·25 c.c. horse serum, H_2O to 50 c.c.
89	1 c.c. S, 40 c.c. H_2O, 2·0 c.c. HCl, 0·25 c.c. horse serum, 1·8 c.c. NaOH, H_2O to 50 c.c.
52	3·39 mg. Hph 2 HCl in 10 c.c. 0·1 N alcoholic HCl = S. 1·2 c.c. S, 50 c.c. H_2O, 1·6 c.c. NaOH, H_2O to 100 c.c.
55–61	3·54 mg. Hph 2 HCl in 0·6 c.c. NaOH, H_2O to 10 c.c. = S.
55	0·6 c.c. S, H_2O to 50 c.c.
57	0·6 c.c. S, 0·5 c.c. NaOH, H_2O to 50 c.c.
61	0·6 c.c. S, 2 c.c. NaOH, H_2O to 50 c.c.
92–96	2·69 mg. Hph 2 HCl, 0·5 c.c. NaOH, H_2O to 10 c.c. = S.
92	0·7 c.c. S, 1 c.c. protalbic acid (5 mg. per c.c. in 0·006 N NaOH), H_2O to 50 c.c.
94	0·9 c.c. S, 40 c.c. H_2O, 2 c.c. HCl, 1·5 c.c. protalbic acid (as for 92), 1·6 c.c. NaOH, H_2O to 50 c.c.

95 as 94 but 1·9 c.c. NaOH.
96 0·7 c.c. S, H_2O to 50 c.c.
24 0·223 mg. Hph 2 HCl in 100 c.c. 0·1 N alcoholic HCl.
213 0·273 mg. Hph 2 HCl in 50 c.c. pyridine.
251–260 1·68 mg. Hph 2 HCl in 10 c.c. 0·1 N. alcoholic HCl = S.
251 0·8 c.c. d.r.g., 0·5 c.c. Sørensen's pH 4·96 sodium citrate buffer, 0·22 c.c. S, 0·22 c.c. NH_3, H_2O to 10 c.c.
256 1 c.c. g.g., 0·8 c.c. d.r.g., 0·22 c.c. S, 0·4 c.c. NH_3, H_2O to 10 c.c.
257 as 256 but 0·36 c.c. NH_3 and 2 c.c. 1·8 p.c. NaCl.
258 as 257 but 0·32 c.c. NH_3.
260 as 257 but 0·30 c.c. NH_3.
142 1·93 mg. Hph 2 HCl in 10 c.c. 0·05 N alcoholic HCl = S. 5 c.c. rabbit globin 0·2 p.c., 0·43 c.c. NaOH, 0·8 c.c. S, H_2O to 50 c.c.
242–248 2·43 mg. Hph 2 HCl in 25 c.c. 0·1 N alcoholic HCl = S.
242 1 c.c. rabbit globin 0·5 p.c., 0·5 c.c. Sørensen's pH 4·96. Sodium citrate buffer, 0·23 c.c. NH_3, 0·25 c.c. S, H_2O to 10 c.c.
244 as 242 but 0·25 c.c. NH_3, 0·5 c.c. Clark's pH 6·0 phosphate buffer.
246 as 242 but 0·18 c.c. NH_3.
248 as 242 but 0·20 c.c. NH_3, 0·5 c.c. Sørensen's pH 4·16 citrate buffer.

PROTOPORPHYRIN.

34 0·363 mg. Pph Na in 100 c.c. 0·1 N alcoholic HCl.
40 1·62 mg. Pph Na in 100 c.c. H_2O.
205 1·85 mg. Pph Na in 10 c.c. H_2O = S.
0·3 c.c. S, pyridine to 10 c.c.
109–116 2·38 mg. Pph Na in 10 c.c. H_2O = S.
109 0·65 c.c. S, HCl to 50 c.c.
116 0·3 c.c. S, N HCl to 50 c.c.
121–125 3·78 mg. Pph Na in 10 c.c. 0·1 N alcoholic HCl = S.
121 0·5 c.c. S, 40 c.c. H_2O, 2 c.c. N HCl, H_2O to 50 c.c.
122 0·7 c.c. S, 40 c.c. H_2O, 0·5 c.c. N HCl, H_2O to 50 c.c.
124 1 cc.. S, 10 c.c. H_2O, 0·5 c.c. horse serum, H_2O to 50 c.c.
125 0·8 c.c. S, 10 c.c. H_2O, 15 mg. protalbic acid in 3·6 c.c. 0·03 N NaOH, H_2O to 50 c.c.
189–194 2·05 mg. Pph Na in 10 c.c. 0·1 N alcoholic HCl = S.
189 4·5 mg. casein, 0·8 c.c. NaOH, 5 c.c. H_2O, 0·5 c.c. S, H_2O to 10 c.c.
191 as 189 but 0·5 c.c. NaOH.
192 0·1 c.c. horse serum. 0·52 c.c. NaOH, 5 c.c. H_2O, 0·5 c.c. S, H_2O to 10 c.c.
194 0·2 c.c. horse serum, 5 c.c. H_2O, 0·7 c.c. NaOH, 1 c.c. Sørensen's pH 7·2 phosphate buffer, 0·5 c.c. S, H_2O to 10 c.c.
230–238 2·07 mg. Pph Na in 10 c.c. alcoholic 0·1 N HCl = S.
230 1 c.c. rabbit globin (0·5 p.c.), 0·22 c.c. NH_3, 0·5 c.c. Clark's pH 6·8 phosphate buffer, 5 c.c. H_2O, 0·22 c.c. S, H_2O to 10 c.c.
233 as 230 but 6·0 buffer.
235 as 230 but 7·6 buffer.
237 as 235 but 0·26 c.c. NH_3.
238 as 235 but 0·35 c.c. NH_3.
264–270 2·02 mg. Pph Na in 10 c.c. 0·1 N alcoholic HCl = S.
264 1 c.c. g.g., 0·8 c.c. d.r.g., 1 c.c. 1·8 p.c. NaCl, 0·5 c.c. NH_3, 5 c.c. H_2O, 0·4 c.c. S, H_2O to 10 c.c.
265 0·8 c.c. d.r.g., 1 c.c. g.g., 1 c.c. 1·8 p.c. NaCl, 0·35 c.c. NH_3, 5 c.c. H_2O, 0·25 c.c. S, H_2O to 10 c.c.
266 as 265 but 0·38 c.c. NH_3.
268 as 265 but 0·4 c.c. NH_3.
270 as 265 but 0·5 c.c. NH_3.
283–291 3·50 mg. Pph Na in 10 c.c. 0·1 N alcoholic HCl = S.
283 0·5 c.c. g.g., 0·5 c.c. Sørensen's pH 4·96 sodium citrate, 0·5 c.c. S, H_2O to 10 c.c.
284 as 283 but with 0·5 c.c. HCl.
286 as 283 but with 0·6 c.c. NH_3.
288 5 mg. protalbic acid, 0·07 c.c. NaOH, 5 c.c. H_2O, 0·7 c.c. NH_3, 0·33 c.c. S, H_2O to 10 c.c.
289 as 288 but 0·65 c.c. NH_3, 0·5 c.c. S.
291 as 289 but 0·9 c.c. NH_3.

THE RAPID ESTIMATION OF THE GOLD CONTENT OF GOLD SOLS

by

S. W. PENNYCUICK AND C. E. WOOLCOCK

(From the Johnson Chemical Laboratories, University of Adelaide).

(Submitted for publication 19th August, 1937.)

Methods for the rapid estimation of the amount of dispersed material in any given sol are in general very rare. Whilst working with gold sols, the authors had occasion to require samples of Bredig gold sols with exactly the same gold content. This entailed a quantitative estimation of the dispersed gold.

The only method available was that of coagulation followed by the gravimetric estimation of the precipitated gold. This has the obvious disadvantage of being rather slow and requiring relatively large quantities of the sol.

As metallic gold is soluble in potassium cyanide, a more rapid and convenient method was developed in which the highly coloured gold sols were titrated to a colourless solution with cyanide and a quantitative measure of the colloidal gold content thus obtained.

The Reaction.

When the pH is kept above 7, the dissolution of gold in potassium cyanide solution proceeds according to the equation:

$$4\,Au + 8\,KCN + 2\,H_2O + O_2 = 4\,KAu\,(CN)_2 + 4KOH$$

The free energy of this reaction is very small, being of the order, — 90,000 calories (Barsky, Swainson and Hedley, 1934), and so the action proceeds almost completely to the right.

By taking a measured volume of gold sol and titrating with a standard solution of KCN, the actual gold content of the sol may be calculated from the above equation, and expressed in milligrams per litre.

The pH of the Sol.

Large-scale practice has shown that the action of cyanide on gold has a maximum velocity over a pH range 10 to 13. As KCN is considerably hydrolysed, a high pH has the further advantage of repressing the hydrolysis and thus preventing the escape of free HCN. This is an important factor in the present work, as the action is a quantitative one. Accordingly all reactions must be carried out in basic solution.

When the pH is too high the gold sol turns blue as a preliminary to coagulation, and on this account all titrations were carried out at values somewhat below 12, the exact value having no great significance. The freshly-formed blue sols at higher pH values give approximately the same titration figures, but as they do not titrate so readily, it is better to avoid them.

The Colour Change.

The red colour of gold sols is remarkably persistent. When a sol containing 100 mg. per litre is diluted in the ratio of 1:100, the colour is still apparent although the diluted sol contains only 1 mg. of gold per litre. It is thus unnecessary to utilize a colorimeter and titrate to some standard colour strength; instead, the titration may proceed until the sol is decoloured. To determine the end point, a diluted gold sol containing 1 mg. of gold per litre and a sample of clear water are used as comparator solutions and the titration continued until the colour lies between these two limits.

The accuracy of observation with a sol containing 100 mg. per litre is thus seen to be within 1 p.c.

Practical Details.

In order to accelerate the reaction, all titrations were carried out at about 100° C., and to ensure a sufficiency of oxygen (which is one of the reactants), the sols were stirred with a slow stream of air or oxygen throughout.

The actual titrations were carried out in small, squat beakers of 25 to 45 c.c. capacity. Precisely 10 c.c. of a sample of gold sol were placed in one of the beakers, and after the addition of NaOH sufficient to raise the pH to 11 or 12, the mixture was brought to the boiling point on a hot plate. It was then removed and titrated to a colour just short of the end point, and replaced on the hot plate for 10 minutes. If the trace of colour persisted a further drop of KCN was added. The procedure was repeated, with the object of ensuring that the minimum amount of KCN was used. The comparator solutions already mentioned were kept on the hot plate and replenished with water when necessary.

As KCN slowly loses its strength, the stock solution was standardized with silver nitrate before being diluted to ·003N and ·006N. These strengths were the most suitable for the titrations.

Accuracy of the Method.

As there was a possibility that a small amount of free HCN would escape from the hot solutions despite the high pH values, a series of titrations was carried out over a wide pH range. In acid solution all sols showed an appreciable loss. In neutral solution the quickly dissolving sols showed no measurable loss, but the slowly dissolving ones were subject to some variation. With all sols, however, there was strict agreement between results over a pH range 9 to 11; so that at this alkalinity there is no appreciable error due to loss of HCN.

The accuracy of the method therefore rests on the determination of the end point, and by the use of comparator solutions this can be measured within an error of 1 p.c. for sols containing 100 mg. or more per litre. In the case of weaker sols the margin of error increases, and a greater degree of accuracy may be obtained by using a colorimeter. For the purpose of the present work, however, the accuracy of from 1 to 2 p.c. obtainable by direct titration was considered sufficient.

Preparation of the Sols.

Five typical gold sols were investigated. Their methods of preparation and their behaviour towards titration are set out briefly below.

(a) *Bredig gold sol in NaOH.* This sol was prepared by arcing pure gold rods in ·002N NaOH in an atmosphere free from CO_2, according to the method used by Pennycuick (1927). The current used was 4 to 5 amp., the voltage 220 volts, and the time of arcing 15 to 20 minutes. As the pH of the sol was 9·98, it required no further base to give concordant results. It titrated just as easily in the cold as when hot, and the fact that no extra oxygen was required indicates a sufficiency of dissolved oxygen to supply the necessary requirements.

(b) *Bredig gold sol in KCl.* This sol was prepared similarly to the preceding one, but the stabilizing electrolyte was ·0004 N KCl, and no precautions to exclude CO_2 were necessary. The pH of the sol was 6·2. It dissolved in KCN more slowly than the former sol, but when heated, stirred with oxygen, with the pH raised to 11, it titrated readily.

(c) *Phosphorus gold sol.* This sol was prepared by the reduction of Merck's crystalline auric chloride, using phosphorus dissolved in ether, according to the method described by Thomas (1934). When titrated in the cold the reaction was slow, but at higher temperatures, although the pH of the preparation was as low as 7·3, the action proceeded rapidly and without the addition of oxygen. When the pH was raised above 11, oxygen had to be bubbled through the sol before the colour was completely discharged. This supports the contention of technical workers that oxygen is less soluble in highly basic solutions.

Observations with the ultra-microscope revealed that the particles were amicrons, and much smaller than the particles of the Bredig sols. As the larger particles dissolve more readily, the rate of solubility in cyanide is evidently not merely a function of particle size.

(d) *Tannin gold sol.* The method used in the preparation of the highly-coloured, finely-dispersed gold sols using tannin as the reducing agent is described by Holmes (1922).

The particles of these sols are also very small, but their rate of solution in KCN is less than that of any of the preceding sols. This again shows the marked influence of surface structure on the process of solution. The most favourable conditions of titration—high pH, high temperature, and a stream of oxygen—must be applied, and the titrated samples must be allowed to stand for 10 minutes on the hot plate to ensure the completion of the reaction.

(e) *Formaldehyde gold sol.* These sols are perhaps the best known of the reduced gold sols, being widely used in the Lange test of cerebrospinal fluids. They were prepared according to the method of Cruickshank (1920).

Numerous samples were prepared all showing pH values around 7·0. Although many differed widely in appearance, all were titratable.

The outstanding feature was the wide variation in the rate of solution, varying from moderately slow in the case of the bright, clear sols to very slow in the case of the "sheeny" or less bright samples. As the clear red sols contain the smaller particles, the order of solubility is in agreement with particle size; but measurements with the ultra-microscope show a complete lack of quantitative agreement between size and solubility. This points to variations in the surface structure of the bright sols on the one hand and the dull or sheeny sols on the other. It is quite probable that this difference in surface structure accounts for the widely different manner in which these types of sol respond to the Lange test, and this matter is at present receiving further attention.

The formaldehyde sols are the slowest in dissolving and therefore the pH values must be kept as high as possible, and they must be kept on the hot plate a little longer than usual.

TABLE 1.

Table of the Gold Content of Various Sols obtained by Titration, by Gravimetric Analysis, and by Calculation.

	Gold content in mg. per litre.		
Type of Sol.	By titration.	By weighing.	By calculation.
Bredig sol (NaOH)	96 mg.	94·8 mg.	—
Bredig sol (KCl)	104 „	103 „	—
Phosphorus gold sol	96 „	94·6 „	95·4 mg.
Tannin gold sol	43·8 „	42·6 „	—
Formaldehyde gold sol	47·5 „	44·6 „	47·7 „

Comparison with Micro-gravimetric Analysis.

In order to compare the titration results with the more usual ones obtained by coagulation and weighing, samples of the sol were taken and coagulated by the freezing method used by Pennycuick (1928, 1929) in the investigation of colloidal platinum. This method of coagulation is preferable to one that entails the addition of coagulating electrolytes, since it leaves the precipitated metal in as undisturbed a state as possible.

From 50 to 75 c.c., according to the gold content of the sol, were coagulated by freezing, giving a coagulum varying in weight from 3 to 5 mg. The thawed sol and the coagulum were then filtered through a micro-Gooch filtering tube as in the micro precipitation analysis outlined by Pregl (1930). The weight of the coagulum was then determined with an accuracy of about 0·5 p.c. using a micro-balance. The results are set out in Column 3 of Table 1.

The figures in Column 4 were calculated on the assumption that the gold chloride had the formula $HAuCl_4$, 4 H_2O, and that all the gold was reduced to the colloidal form. This calculation could be applied only to the phosphorus and formaldehyde reduced sols.

Comments on the Results.

On comparing the titration with the gravimetric results, the outstanding feature is the slightly greater values shown by the former method. In this method one would expect maximum or full values, seeing that the whole of the colloidal gold is titrated, whereas in the gravimetric method it is conceivable that some small fraction of the gold escapes coagulation by the freezing process, in which case this method falls a little short of 100 p.c. efficiency. The authors are of the opinion that the titration results are the more reliable, and this view is supported by the fact that the calculated results (Column 4) agree very closely with those obtained by titration.

Advantages and Applications.

The titration method, apart from its accuracy, has two distinct advantages, namely rapidity of estimation and small amount of sol required. The estimation, with the rechecking, can be carried out in 30 minutes, whilst the total sol required, again allowing for rechecking, amounts only to 50 or 60 c.c.

This method will be found convenient whenever it is required to prepare sols of the same total gold content. It will also prove useful, as the authors have already found, in determining the size of the particles in any given sol, where, apart from the ultra-microscopic count, the gold content of the sol must also be determined.

In the routine estimation of gold, or in the determination of the purity of a gold salt, or of the amount of dissolved gold in a given solution, it is possible to utilize this method by first converting the gold quantitatively into the formaldehyde sol. The authors are developing rapid and simplified methods of preparing formaldehyde sols, and it is by no means inconceivable that this method of analysis will ultimately prove of some general use.

SUMMARY.

A simplified method of measuring the gold content of gold sols has been developed, namely the direct titration of the sol with potassium cyanide. The method is very convenient and is apparently applicable to all gold sols. The conditions of titration and the accuracy of the method have been determined for the following sols: neutral Bredig sol, alkaline Bredig sol, phosphorus reduced sol, tannin reduced sol, formaldehyde reduced sol.

ACKNOWLEDGMENT.

The authors are indebted to the Trustees of the Endowment Fund of the Council for Scientific and Industrial Research of the Commonwealth of Australia for a grant for the purchase of the gold used in this work.

REFERENCES.

Barsky, G., Swainson, S. J. and Hedley, N. (1934): Amer. Inst. of M. & M. Engineers, 112, p. 660.
Cruickshank, J. (1920): Brit. J. exp. Path., 1, p. 71.
Holmes, H. N. (1922): "Laboratory Manual of Colloid Chemistry", John Wiley, New York, p. 13.
Pennycuick, S. W. (1927): Austral. J. exp. Biol., 5, p. 199.
Pennycuick, S. W. (1928): J. Chem. Soc., Lond., p. 2108.
Pennycuick, S. W. (1929): *Ibid.*, p. 618.
Pregl, F. (1930): "Quantitative Organic Microanalysis", Churchill, London, p. 124.
Thomas, A. W. (1934): "Colloid Chemistry", McGraw-Hill, New York, p. 110.

STUDIES ON THE LIFE HISTORY AND ECONOMIC IMPORTANCE OF *HETERAKIS GALLINAE* (GMELIN, 1790 FREEBORN, 1923), THE CAECUM WORM OF FOWLS

by

F. H. S. ROBERTS

(From the Animal Health Station, Yeerongpilly, Brisbane).

(Submitted for publication 2nd July, 1937.)

INTRODUCTION.

The round worm, *Heterakis gallinae*, which infests the caeca of poultry and game birds, has a world-wide distribution and has been recorded from 33 different kinds of birds (Clapham, 1933b). Observations in the U.S.A. show that from 62 to 74 p.c. of the domestic fowls in an area may be infested [Ackert (1917), Riley and James (1921), Dorman (1928)]. In Natal, Le Roux (1927) found it in each of 124 birds examined. In England, 75 to 80 p.c. of the fowls examined were found to be infested [Taylor (1929), Clapham (1933b)].

H. gallinae was first recorded from Australia by Cobb in 1896. It is known to occur in Queensland, in the fowl, duck and turkey; Baylis (1934) also recorded it together with *H. isolonthe* from an introduced Amherst pheasant (*Chrysolophus amherstiae*). During 1932, the writer found that 94·6 p.c. of 128 domestic fowls examined in the Brisbane area were infested with *H. gallinae*. During the present investigations, 89·7 p.c. of 616 birds examined in this area were found to be infested.

The number of worms present in infested birds varied from 1 to 1212; the average number from 546 birds examined was 41·8. From 1–9 worms were found in 46 p.c. of the infested birds, 10–100 in 42·5 p.c., 101–500 in 9·9 p.c., 501–1,000 in 1·3 p.c. Only one bird contained over 1,000 worms. Dorman records 1–284 worms from 95 birds, giving an average of 45 per bird; Ackert obtained 1–326 worms from 395 birds, an average of 34 per bird; Riley and James obtained 1–113 worms from 273 birds. Clapham (1934a) found that the majority of the birds examined contained only 20–30 worms or less; the number of worms rarely exceeded 100.

The investigations described in this paper were undertaken primarily to obtain information about the pathogenicity of *H. gallinae*. The work was seriously hampered by the occurrence, in the majority of the birds, of "blackhead" disease,

which is transmitted by the worm parasite (Tyzzer, 1934). The birds used in the experiments were selected as day-old chicks and safeguarded against infection with *H. gallinae* until they were 40 days old. At this age, infective eggs of the worm were fed to the birds; in Groups 1 and 2 (white leghorns), single doses of 280 eggs and 1,400 eggs respectively were given; in Group 3 (Australorps) a single dose of 3,000 eggs was given.

LIFE HISTORY OF *H. GALLINAE*.

The life-cycle of *H. gallinae* is direct and does not involve any extra-intestinal migration as does the life cycle of *Ascaris lumbricoides*. Opinions vary, however, as to whether or not the young worms invade the caecal tissues during the early stages of their development. There are also marked discrepancies in the recorded periods for the development of the egg to the infective stage, and for the worms to reach maturity. Certain observations on the life history, made by the writer, are recorded here, as they may assist in clearing up some of these discrepancies. The possible influence of "blackhead" on the development of the parasite will be discussed in a later section.

(1) *The Preparasitic Life Cycle.*

The optimum temperature for the development of the egg is 33° C. At this temperature and under 2 mm. of 1 p.c. formalin, the first larval moult occurs in the egg on the fourth day; at 27° C. it occurs on the sixth day. Trials showed that the larvae are infective within 24 hours after moulting.

The infective or second stage larva is enclosed in the moulted skin of the previous stage; it is characterized by an oral chitinous prominence, which is uneven in outline, so that the mouth appears subterminal. A pair of faint, lateral striae is also present, from which the lateral membranes of the later stage larvae are developed.

(2) *The Parasitic Life Cycle.*

Free larvae may be found in the intestine of chicks two hours after giving them eggs containing infective larvae. They are most numerous in the region lying between the entrance of the bile ducts and the remnant of the yolk sac. Their length varies from 269μ to 335μ, and averages 302μ. Within 4½ hours the larvae have reached the ileum and two hours later a few may be found in the caeca. At the end of 24 hours the majority are present in the caeca; a few may still be present in the ileum, where they may persist to about the fifth day. On reaching the caeca, the larvae immediately invade the mucosa. During the first two days this is so marked that no, or very few, larvae are recovered from the lumen by washing the caeca and examining the washings. After the second day, the larvae begin to reappear in the lumen, their association with the tissues becoming less pronounced, but persisting to about the fifth day. Sections show that, during this

period, the larvae invade the epithelium of the crypts, occasionally reaching the reticular tissue (Fig. 1). Some may only partly invade the epithelium, portion of the body remaining in the crypt (Fig. 2). Usually, however, penetration into the epithelium is complete, and the larva lies in the tissues coiled around the crypt (Fig. 3). After the fifth day, an occasional worm may penetrate into a lymph gland but, at this time, they are in the lumen and lying in the mucus adjacent to the mucosa.

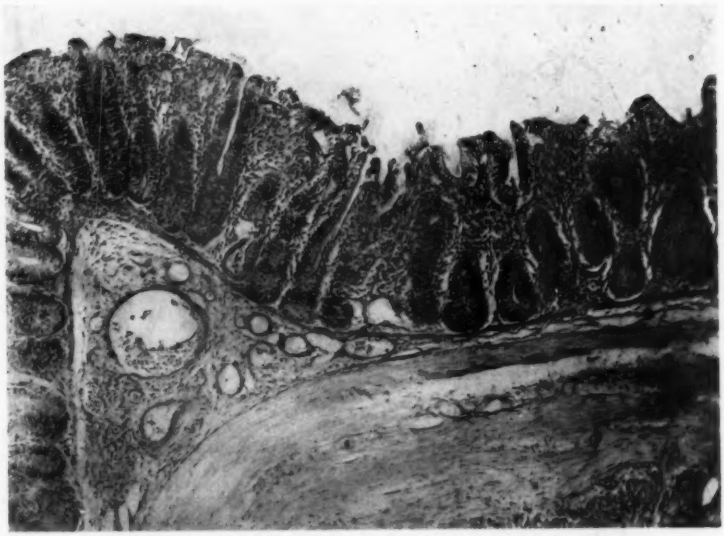


Figure 1. *Heterakis gallinae.* Forty-eight hours after infestation, showing larva partially embedded in the reticular tissue of the caecum.

The second moult occurs on the 4th to 6th day. The third stage larva is characterized by three shallow and indistinct lips, prominent lateral membranes and a conspicuous genital primordium. The chitinous, oral prominence seen in the previous stage has entirely disappeared. At the age of seven days larvae may measure up to 2·10 mm. in length. During the succeeding days, traces of the lip papillae may be detected. In the females, a rudimentary vagina is present and in the males, which are shorter and more stumpy than the females, a preanal swelling, the rudiment of the preanal sucker, can be distinguished.

The third moult was observed on the 9th to 10th day, and at this time worms up to 3·87 mm. in length may be present. The male now possesses a prominent preanal swelling, chitinization of the spicules has commenced, and traces of some of the caudal papillae can be seen. In the female, the vagina is conspicuous and a

cuticular swelling marks the appearance of the vulva. In both sexes the three lips surrounding the mouth with their papillae are apparent.

The final moult occurs about the 14th day. In both sexes the lips and oesophagus are now of the adult type; in the male, the preanal sucker, all caudal papillae and the spicules are present. Twenty-four days after infestation, female worms, 6·72 to 7·3 mm. in length, with the uteri filled with fertile eggs, are present.

Figure 2. *Heterakis gallinae.* Twenty-four hours after infestation, showing larva attacking the epithelium of a caecal crypt. Penetration is incomplete, portion of the larvae remaining in the lumen of the crypt.

(3) *Comparison with Observations by Previous Authors.*

(a) *Period Required for the Egg to Develop Infective Larvae.* Graybill found that eggs are infective in about 7 days at 29° C., whilst Uribe, Riley and James, and Dorman give 9–11 days. Dorman gives the optimum temperature for development as 33° C. Clapham (1933b), however, states that the temperature

range for development is 20° to 30° C., the optimum being 26° C., at which temperature eggs develop infective larvae in 14 to 17 days.

Our observations show the optimum temperature for development to be 33° C., at which temperature eggs are infective in 5 days. The period noted by Uribe and Riley and James was recorded at room temperature and "under normal summer conditions" respectively. The difference between their figures and those of Graybill and ourselves is slight and could possibly be accounted for by fluctuating temperatures. It is difficult, however, to find an explanation for the figures quoted by Clapham, for at 27° C. we found infective larvae present in the eggs after 7 days.

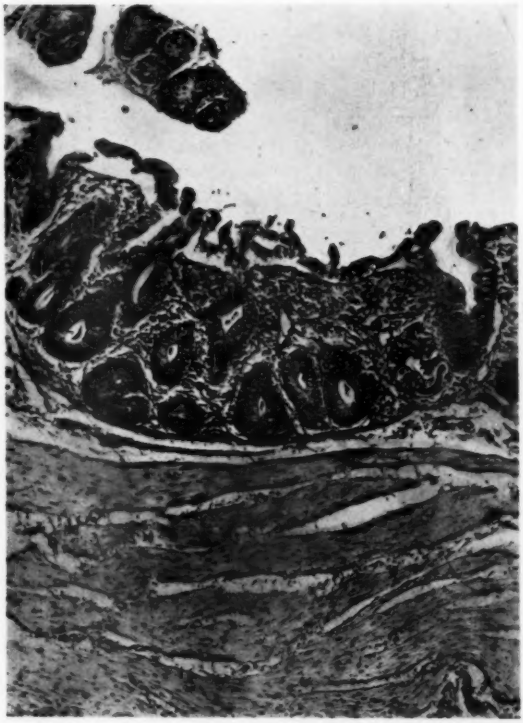


Figure 3. *Heterakis gallinae.* Twenty-four hours after infestation showing larva lying in the epithelium of a caecal crypt, coiled around the lumen. Note the destruction of the epithelium lining the crypt.

(b) *Length of Newly-hatched Larvae.* Graybill gives the length of newly-hatched larvae as 290μ to 340μ; Dorman, 230μ to 255μ; the writer, 269μ to 335μ. Uribe gives an average length of 180μ, Clapham (1933b) 235μ, the writer 302μ.

Baker (1933) gives "about three times the length of the egg case". The estimated measurements on this basis would be 210μ to 250μ.

Whilst the measurements recorded by Dorman, Baker and Clapham agree substantially, those given by ourselves are much greater and agree with those noted by Graybill. The average length quoted by Uribe is not confirmed by any other investigator.

(c) *Location of Larvae during the Early Phases of the Parasitic Life Cycle.* Both Uribe and Baker (1933) state that during the first four days of its parasitic life, the larva invades the epithelium of the glandular crypts. Tyzzer, whilst agreeing that the young worms invade the caecal epithelium, records that they do not return to the lumen till about the tenth to twelfth day. Graybill found a few larvae in the mucosa during the first three days, but the majority were in the lumen. Itagaki (1930) noted that larvae do not as a rule enter the caecal wall, but some may invade the glands, in which they may mature. Clapham (1933b), however, maintains that the worms do not invade the caecal tissues at all but remain free in the lumen throughout the entire period of their parasitic life.

Our own observations agree with those of Uribe and Baker, but we also agree with Itagaki in that we found an occasional worm in the lymph glands.

(d) *Number of Moults in the Parasitic Life Cycle.* Graybill recorded two moults on the 9th to 10th and 16th days respectively; and Uribe two moults on the 9th and 13th days. Baker (1933) has described the final moult as occurring over the 15 to 22 day period, whilst Clapham observed three moults on the second, fourth and tenth day respectively.

We have observed three moults, namely, the second on the 4th to 6th day, the third on the 9th to 10th day and the fourth on the 14th day. Evidently the two moults recorded by Graybill and Uribe correspond to our third and fourth. We could not confirm the moult on the second day as recorded by Clapham, and are of the opinion that the moult noted by her on the tenth day is really the third and not the fourth or final moult as considered by her.

(e) *The Influence of "Blackhead" upon Development of the Larvae.* Observations by previous workers show marked discrepancies in the recorded lengths of larvae of the same age and also in the periods taken by the worms to reach maturity. Clapham and Dorman, for example, saw mature worms in 24 days and 26 days respectively, whilst Graybill and Uribe say that maturity is not reached for 56 to 61 days. Baker (1933) considered these discrepancies to be due to the presence of "blackhead" disease (*Histomonas meleagridis*) which not only retards the development of the worms but also reduces their numbers. Baker observed that in "blackhead"-free chickens, the worms frequently become mature in less than 30 days. Neither Clapham nor Dorman encountered "blackhead" outbreaks in their experimental birds, but Graybill and Uribe frequently saw it.

We have attempted to trace the influence of "blackhead" on the development of the worm, and to compare our findings with those of Baker. The presence or

absence of "blackhead" infection in the caeca was determined by macroscopic changes and by microscopic examination of smears and sections.

The data relating to the numbers of worms found in each bird at autopsy and their measurements are set out in Table 1.

TABLE 1.

Group 1. 280 Eggs.

Bird No.	Period of Infestation, in days.	No. worms recovered.	Length of worms in mm. Range.	Average.
602	1	46(x)	·280– ·337	·308
603	2	69	·318– ·353	·335
604	4+	118	·487– ·790	·59
605	7+	186	1·13 –2·10	1·8
606	9+	64	2·54 –3·87	3·07
607	11+	11	2·14 –3·10	2·60
608	14+	—	— – —	—
609	14+	11	2·25 –3·75	2·82
610	16+	4	·89 –2·5	1·70
611	24—	6	5·26 –7·2	6·40
512	24—	2	6·04 –7·3	6·67
613	24+	—	— – —	—

Group 2. 1,400 Eggs.

Bird No.	Period of Infestation, in days.	No. worms recovered.	Length of worms in mm. Range.	Average.
628	1	120(x)	·269– ·328	·304
629	3+	340	·378– ·495	·452
630	5+	628	·812–1·14	·98
631	8+	366	1·40 –2·72	1·59
632	10+	420	1·40 –3·54	2·41
633	12+	10	·48 –2·68	1·25
634	15+	147	3·02 –5·0	3·78
635	17+	4	·90 –3·12	2·10
636	18+	19	1·80 –4·2	3·18
637	19+	115	3·73 –4·68	3·76
638	24+	9	3·20 –4·0	3·50
639	24+	2	3·10 –3·30	3·20

Group 3. 3,000 Eggs.

Bird No.	Period of Infestation, in days.	No. worms recovered.	Length of worms in mm. Range.	Average.
640	4+	769	·431– ·835	·582
641	5+	597	·496– ·840	·621
642	7+	1,194	·610–1·18	·76
643	7+	369	·90 –1·54	1·20
644	8+	1,575	·82 –2·42	1·65
645	9+	1,060	1·30 –2·97	2·10
646	9+	516	1·39 –2·60	2·02
647	10+	199	2·02 –3·33	2·81
648	11+	123	·94 –3·04	2·33
649	11+	8	·57 –1·89	1·35
650	11+	160	·56 –2·21	1·36
651	11+	1,100	1·36 –3·65	3·0
652	11+	136	·89 –2·44	1·80
653	12+	181	1·73 –3·46	2·54
654	14+	—	— – —	—
655	14+	6	2·27 –3·27	2·87
656	14+	16	1·58 –2·87	2·50
657	15—	359	3·54 –4·9	4·4
658	15+	3	·62 – ·75	·69
659	19+	45	·51 –1·88	1·04
660	22+	—	— – —	—
661	23—	180	5·29 –6·88	6·30
662	25+	1	5·9	5·9
663	25+	21	1·9 –2·54	2·31

(x) Note: The larval counts during the first five days are by no means accurate as during this period many of the larvae are present in the mucosa.

Note: + = "Blackhead" present. — = "Blackhead" absent.

In Group 1 all birds were affected with "blackhead" except Nos. 611 and 612. In Group 2 no birds were free from this disease, but in Nos. 634 and 637 the infection was light. In Group 3, Nos. 657 and 661 were not affected, whilst No. 662, in the caecum from which a single worm was secured, was but lightly infected.[1]

The table shows that only in those birds which were either entirely free from "blackhead" or were only lightly infected with this disease, did the worms make normal growth. In the remaining birds, which were severely infected, the growth of the worms was considerably retarded.

Apart from the possible influence of "blackhead" on the survival of larvae, it is apparent that the percentage of surviving larvae may occasionally be very low even in healthy caeca. For instance, in Group 1, which was fed 280 eggs, only 6 and 2 larvae had survived in Nos. 611 and 612 respectively, these being the only two birds of this group in which the disease did not appear. In Group 2, however, greater numbers of larvae survived in the lightly infected birds (Nos. 634 and 637) than in the severely infected birds, autopsied after similar periods of infestations. The same phenomenon is encountered in Group 3, when the larval survivals in birds Nos. 657 and 661 are considered.

In each group, many larvae were present and were making progressive growth up to about the 10th day. The growth of larvae in healthy birds as given by Clapham (1933b) shows, moreover, that even though these larvae occurred in birds affected with "blackhead", their growth at any time during this ten-day period, compares very favourably with that of larvae in healthy birds. After about the tenth day, however, there was a conspicuous diminution in the numbers of the larvae, and thereafter, except in "blackhead"-free birds, the growth rate of the larvae was erratic and, in general, retarded. Graybill also noted that, after a certain period of infestation, a conspicuous diminution in the numbers of the larvae may take place, but in his experiments this decrease did not become apparent till the 16th day. In both Graybill's and the writer's experiments "blackhead" outbreaks occurred, but as the survival of larvae is usually very low even in healthy birds, it cannot be said with any degree of certainty that this sudden and marked decrease in the numbers of the larvae is associated solely with the presence of "blackhead". It is considered possible, however, that in healthy birds, the elimination of the larvae is a gradual process, whilst in birds affected with "blackhead" the disease may eventually reach a stage at which it is more unfavourable to the larvae than at any previous time.

PATHOGENICITY.

Opinions regarding the pathogenicity of *H. gallinae* vary. Baker (1929) considers the presence of the worms is necessary for the efficient functioning of the

[1] According to Tyzzer (1934), the organism of blackhead does not invade the mucosa till about 3 days after infection. In these observations, therefore, only those birds infested for 3 days or longer are considered.

caeca. Clapham concludes that whilst the worms are not beneficial to the host, they are also not harmful. Uribe, after discussing the damage by the larvae to the mucosa and by the grown worms which occasionally attach themselves to the mucosa and even invade the lymph glands, says, "There is, however, no evidence that the parasite produces very serious injury to the mucosa".

Figure 4. *Heterakis gallinae.* An adult worm attacking a caecal lymph nodule. The lymph tissue has been partially destroyed with conspicuous haemorrhage.

Riley and James, Van Es and Martin (1923), and Beach and Freeborn (1930), however, consider the worms may be responsible for a typhlitis, interfering with the normal growth of young birds and sometimes causing death.

Nodule formation in the caecal wall has been recorded by Létulle and Marotel (1909) but is considered by Schwartz (1925) to be caused only by *Heterakis isolonthe* and *H. beramporia.* Itagaki, however, has reported nodules in the caeca of naturally infested birds at Tokyo, Japan, but was unable to produce them in experimental birds.

The chief economic importance of *H. gallinae* is associated with its rôle as a vector of "blackhead" in turkeys and chickens [Graybill and Smith (1920) and

Tyzzer (1934)]. The question of the pathogenicity of this helminth may therefore be complicated by the typhlitis which "blackhead" itself produces. Tyzzer, however, considers that heavy infestations of developing worms may produce *per se* a characteristic helminthic typhlitis, which is caused by the young worms "growing out of" the glands invaded during the early phases of the life cycle.

We have examined caeca from nearly 700 birds, but obtained little information regarding any pathogenicity; although lesions were often present, these could not be directly associated with the presence of worms. The only constant feature of moderate to heavy infestations was a thickening of the caecal apices, where the worms frequently congregate. Nodule formation was absent. Enlarged lymph glands were, however, frequently seen, but as these occurred both in infested and non-infested birds, their association with *H. gallinae* was by no means apparent. In some instances these glands had been invaded by large worms, the whole parasite or just the anterior end being buried in the gland with varying degrees of haemorrhage and tissue destruction (Fig. 4). Inflammation of the caecal mucosa was neither conspicuous nor constant, even with heavy infestations (500–1,000 worms) and there appeared to be little correlation between the presence or degree of inflammation and the number of worms present. Apparently, however, a very heavy infestation may be associated with an inflamed mucosa, for the writer (1932) has recorded three cases of marked typhlitis in which infestations of 2,030, 1,862, and 1,679 worms respectively were present in old hens.

In the experiments in which single doses of 280, 1,400 and 3,000 eggs were employed, any pathogenic effects such as might be detected by an examination of the caeca or from the appearance of the birds, were masked by the presence of "blackhead", which disease was present in most birds. The invasion of the caecal mucosa by the larvae during the first few days of infestation was accompanied by the destruction of epithelium (Figs. 1, 2, 3); later on, the caeca of the few chickens which did not develop "blackhead" were entirely normal in appearance. No changes could be detected on microscopic examination, except in those areas from which the epithelium had been eroded by the early stages in development.

It may be concluded, therefore, that *Heterakis gallinae* is of considerable economic importance as a vector of the organism of "blackhead" or enterohepatitis. In the absence of this disease, however, the worm does little harm except on rare occasions when it is present in very large numbers.

SUMMARY.

In the Brisbane district, although heavy infestations are rare, 89·7 p.c. of all fowls examined harboured *H. gallinae*. At 33° C., the optimum temperature for development of *H. gallinae*, eggs develop infective larvae in 5 days, the first moult occurring on the 4th day. There are three moults in the parasitic life cycle, namely, on the 4th to 6th, 9th to 10th and about the 14th day respectively. Matu-

rity is reached in 24 days. The young larvae invade the caecal epithelium of the host soon after infection, but within 2 to 5 days return to the lumen of the bowel where they complete their development.

The relationship between *H. gallinae* and "blackhead" is discussed. In the absence of "blackhead", the worms are not harmful to the host, unless present in very large numbers.

ACKNOWLEDGMENT.

We wish to record our thanks to Mr. I. W. Helmsing, Illustrator, Department of Agriculture and Stock, for the photomicrographs accompanying this article.

REFERENCES.

Ackert, J. E. (1917): Science, 46, p. 394.
Baker, A. D. (1929): Poult. Sci., 8, p. 59.
Baker, A. D. (1933): Sci. Agric., 13, p. 356.
Baylis, H. A. (1934): Parasit., 26, p. 129.
Beach, J. R. and Freeborn, B. (1930): "Diseases and Parasites of Poultry in California", Agri. ext. Service, Univ. California, Circ. 8.
Clapham, P. A. (1933a): J. Helminth., 11, p. 9.
Clapham, P. A. (1933b): *Ibid.*, 11, p. 67.
Clapham, P. A. (1934a): *Ibid.*, 12, p. 71.
Clapham, P. A. (1934b): *Ibid.*, 12, p. 123.
Cobb, N. (1896): Agric. Gaz. N.S.W., 7, p. 748.
Dorman, H. P. (1928): Trans. Amer. micros. Soc., 47, p. 379.
Graybill, H. W. and Smith, T. (1920): J. exp. Med., 31, p. 647.
Graybill, H. W. (1921): J. exp. Med., 34, p. 259.
Itagaki, S. (1930): Rep. 4th World's Poult. Congress, London, p. 481.
Le Roux, P. (1927): 11th and 12th Rep. Dir. vet. Educ. and Research. Pretoria, p. 209.
Létulle, M. and Marotel, G. (1909): Arch. Parasit., 12, p. 361.
Riley, W. and James, L. G. (1921): J. Amer. vet. Med. Assn., 59, p. 208.
Roberts, F. H. S. (1932): Queensland Agric. J., 38, p. 344.
Schwartz, B. (1925): Phillipine J. Sci., 28, p. 1.
Taylor, E. L. (1929): J. Minis. Agric., 36, p. 533.
Tyzzer, E. E. (1934): Proc. Amer. Acad. Arts and Sci., 69, p. 189.
Uribe, C. (1922): J. Parasit., 8, p. 167.
Van Es, L. and Martin, H. H. (1923): "The More Important Poultry Diseases", Agric. exp. Stat., Univ., Nebraska, Bull. No. 195.

CIRCULATORY EFFECTS OF THE VENOM OF THE INDIAN COBRA *(NAIA NAIA)* IN DOGS

by

W. FELDBERG AND C. H. KELLAWAY

(From the Walter and Eliza Hall Institute, Melbourne).

(Submitted for publication 11th August, 1937.)

Most of the early workers who have studied the action of the venom of the Indian cobra (*Naia naia*) on the arterial blood pressure have concentrated their attention on the central effects of the venom and on its toxic action on the heart. Little work has been done on those peripheral effects of the venom which may be related to its cell-injurious action, though *post mortem* in animals and men poisoned by the venom haemorrhagic lesions have been observed. Only recently has a depressor action of cobra venom, caused by peripheral vasodilatation, been described in man (Vernes and Koressios, 1934) in the dog (Gautrelet and Halpern, 1934; Gautrelet, Halpern and Corteggiani, 1934) and in the rabbit (Laignel-Lavastine, Würmser and Koressios, 1934).

We have shown (1937a) that histamine is liberated by cobra venom from the perfused lungs of cats and guinea-pigs, and have studied the circulatory effects of the venom on the cat (1937b) with special reference to the part played by histamine in their production. The immediate fall in blood pressure in this species is the outcome of obstruction in the pulmonary circulation. The present paper is concerned with the circulatory effects of cobra venom in the dog. We were able to confirm the observation of Gautrelet and his co-workers, that cobra venom exerts a powerful depressor action in this species, and have sought to ascertain what part heart failure, peripheral vasodilatation and obstruction in the pulmonary and hepatic circulations play in causing this effect, and whether it is attended by loss of fluid from the circulating blood.

METHODS.

The dogs (6–16 kg.) after a subcutaneous injection of morphine (10–15 mg. per kg.) were anaesthetized with chloroform and then given 100–110 mg. per kg. of chloralose intravenously. In experiments lasting more than three hours it was often necessary to inject a second dose of chloralose. The vagi were divided, a cannula was tied into the trachea, artificial ventilation established and the arterial blood pressure recorded from a carotid artery. The venom was injected either intravenously through a cannula tied into the femoral vein, or intraportally through a cannula tied into the central stump of a mesenteric vein. For this purpose the loop of intestine corresponding to the vein was removed. In the experiments in which the venous pressure was

recorded a cannula was tied into the right external jugular vein and connected through a water manometer (filled with saturated sodium sulphate solution) to a Brodie's tambour.

For studying changes of pressure in the pulmonary artery the thorax was opened on the left side by resection of portions of two or three ribs. The left lung was removed and a cannula tied into its artery, connected to a mercury manometer by a rubber tube and filled with saturated sodium sulphate solution. In order to secure a good length of vessel for the insertion of the cannula, the arterial branches supplying the apical lobe were dissected free and tied separately.

The effects on the liver and on the portal circulation were studied by recording the pressure in the portal vein and measuring the lymph flow from the thoracic duct. The pressure in the portal vein was recorded by means of a Brodie's tambour connected through a water manometer to a cannula tied into the splenic vein. The spleen was removed for this purpose. The cannula and manometer were filled with saturated sodium sulphate solution. The lymph was collected from a cannula tied into the thoracic duct near its termination in the neck. Ten to twenty minutes before the cannula was tied in, the blood and lymph were made incoagulable by the intravenous injection of 1·5–2 mg. per kg. of heparin.

The effects on the vessels (hind limb and intestine) were studied using the method described by Feldberg and Schilf (1930). The blood was rendered incoagulable by the intravenous injection of heparin and chlorazol fast pink. The venous outflow was measured with a tipping recorder and then returned to the animal by a vein; the venom, in a volume of 0·2 to 0·4 c.c. of saline, was injected into the artery supplying the organ through a metal cannula (made from a hypodermic needle) tied into the central stump of a side branch of the main artery. The venous outflow from the limb was collected from the femoral vein and returned into a jugular vein. The venom was injected into the central stump of the profunda femoris. In the case of the intestinal vessels, we removed the spleen, large intestine, duodenum and the small intestine with the exception of one loop of ileum about 25 cm. in length. The venous outflow from this loop was returned to the animal through a cannula tied into the splenic vein. All the branches of the superior mesenteric artery were tied near their origin, with the exception of that supplying the loop and the branch distal to it, which was used for injection.

Loss of fluid from the circulating blood was measured by determining, by the Sahli method, the haemoglobin percentage of blood samples taken from the femoral artery (for details see Feldberg and Kellaway, 1937c).

RESULTS.

The intravenous injection of 1 mg. per kg. of venom into the artificially ventilated dog is followed, after a latent period of 20–25 seconds, by a sharp fall of arterial blood-pressure. The fall may be preceded by a slight rise as in the experiment of Fig. 1 (A). If recovery takes place and a second similar dose of venom is injected, there is a sharp rise which may be followed by a slight fall in pressure (Fig. 1 C). When smaller doses are injected intravenously the depressor action of the first is less pronounced and a second dose has no further action on the blood-pressure. When doses larger than 1 mg. per kg. are injected the first initial pressor response may be somewhat larger, the fall of pressure steeper and recovery from it may fail to occur. If recovery takes place, as in the experiment of Fig. 2, the blood-pressure tracing may show Traube Hering waves, despite the fact that the blood appears to be well oxygenated. Traube Hering waves may also occur after repeated injections of smaller doses of venom (Fig. 1 C, and Fig. 6 E). In the

later stages after large doses of venom, cardiac irregularities are a constant feature and alter the blood-pressure tracing characteristically (Fig. 2). In spite of cardiac disturbances the blood-pressure may remain at a high level for some hours or may suddenly fall. A second injection of a large dose of venom (2 mg. per kg. or more) always produces a sharp rise, followed after a few minutes by a steep final fall of pressure.

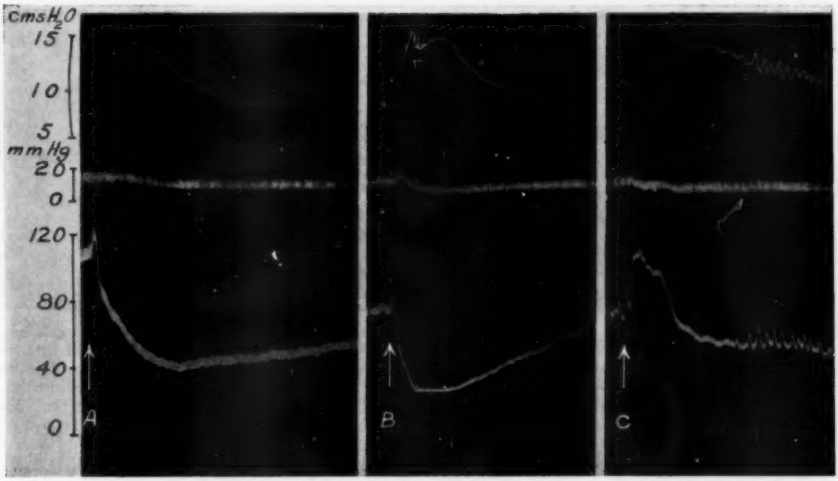


Figure 1. Pressure in the portal vein (upper tracing), in the pulmonary artery (middle tracing) and in the left carotid artery (lower tracing) from a dog weighing 7·5 kg. At A and C, 1 mg. per kg. of cobra venom, at B, 0·027 mg. per kg. of histamine hydrochloride injected intravenously. Time in half minutes.

We found that the initial fall in systemic pressure is not the result of cardiac failure, nor of obstruction in the pulmonary circulation but is caused by vasodilatation, particularly in the parieties, accentuated by the effect of the venom on the liver and by loss of fluid from the circulating blood. The terminal fall in systemic pressure is accounted for by heart failure. The initial transitory rise of pressure is the result of vasoconstriction in the splanchnic area. The pressor effect of a second dose of venom is caused by general vasoconstriction. These conclusions are derived from the following experiments.

EFFECT ON THE HEART.

Venous Pressure.

The initial fall in arterial blood pressure after the intravenous injection of large doses (1·0 to 2·0 mg. per kg.) of cobra venom is invariably accompanied by

a fall of venous pressure (Fig. 2) and cannot therefore be accounted for by failure of the heart. At a later stage these large doses may lead to heart failure which is preceded or accompanied by a rise in venous pressure (Fig. 2). A rise in venous pressure resulting from heart failure also accompanies the terminal fall of arterial blood pressure, which follows closely upon the injection of a second large dose of venom.

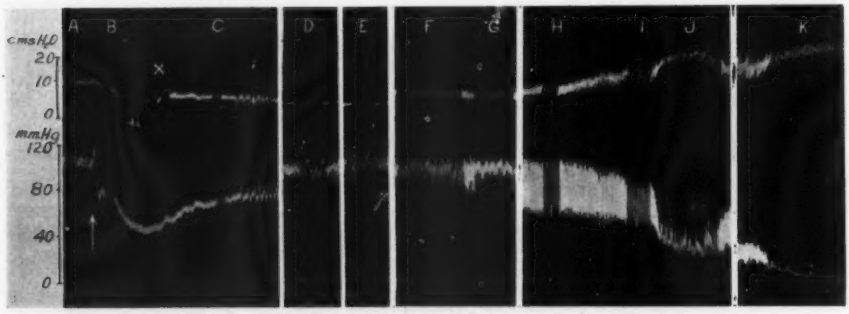


Figure 2. Pressure in the right external jugular vein (upper tracing) and in the left carotid artery (lower tracing) of a dog weighing 11·1 kg. At the arrow (↑) 2 mg. per kg. of cobra venom injected intravenously. The kymograph was driven faster while the electrocardiograms of Fig. 3 were being taken at A to K. The irregularities in the tracing of the venous pressure at (x) were the result of kinking of the vein. Time in half minutes.

Some of the changes in the heart's action occurring at varying periods after the injection of venom can be seen on the record of the arterial blood pressure. Soon after the injection, when the blood pressure has fallen, the heart may beat faster for some minutes; this may be followed by a sudden slowing of rate to half or a third with an increase in the pulse pressure. This stage is sometimes preceded by a period in which irregularities are observed. The slowing of the heart may persist until just before death, or may be interrupted by the sudden onset of tachycardia.

Electrocardiograms.

Gautrelet, Halpern and Corteggiani (1934) have observed in electrocardiographic studies that the injection of 0·1 to 0·2 mg. per kg. of cobra venom in the dog is followed by the appearance of extra systoles. We have analysed the cardiac changes produced by the intravenous injection of large doses of venom (1·5–2 mg. per kg.) by electrocardiograms taken from the right shoulder and left groin (lead 2). Though the changes in the electrocardiograms were somewhat different in every experiment, they all showed impairment of conduction as the dominant feature of the action of the venom. We have not determined whether this is the

result of the toxic action on the conducting tissue or whether it is brought about indirectly by interference with the coronary circulation.

The first changes to be noted, occurring within a minute or two after the injection are in the form of the T. wave. Next the P-R interval becomes progressively longer and the ventricular complexes wider. The development of aberrant ventricular complexes may follow, sometimes preceded by a great diminution in the voltage of the ventricular complexes, or an abrupt onset of heart block occurs.

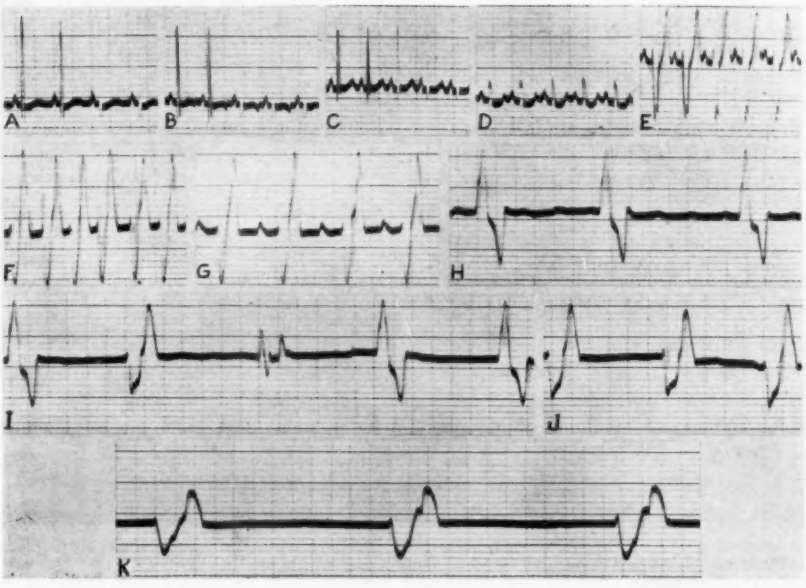


Figure 3. Electrocardiograms (lead 2) from the dog of Fig. 2. At A, B, C and E, the first two ventricular complexes have been retouched for reproduction (for details see text).

This may originally be partial, 2:1 or 3:1, but usually becomes complete. The toxic effects of the venom on the auricles are longer delayed than those on the conducting tissues and on the ventricles. The ventricular complexes are affected earlier than the auricular complexes, which are often difficult to observe because they are buried in the ventricular complexes. In the late stages of block the auricular complexes are reduced in voltage and sometimes disappear altogether.

In the experiment of Fig. 2, the electrocardiograms of which are shown in Fig. 3, the normal T. wave had an upward convexity (A). Immediately after the injection, (B) the wave became diphasic and a few minutes later (C), it showed

an upward concavity with a sharp upward peak. At D, 21 minutes after the injection, the ventricular complexes were wider and of low potential and the P-R interval was lengthened. At E, 7 minutes later, the ventricular complexes were aberrant with increased widening and there was further lengthening of the P-R interval. At F, 48 minutes after the injection, there was tachycardia with ventricular complexes in which the P. waves were buried. At G, a minute later, there was 2 : 1 block which after 12 minutes had changed to complete block at H. The auricular complexes were now greatly reduced in voltage and later than this were

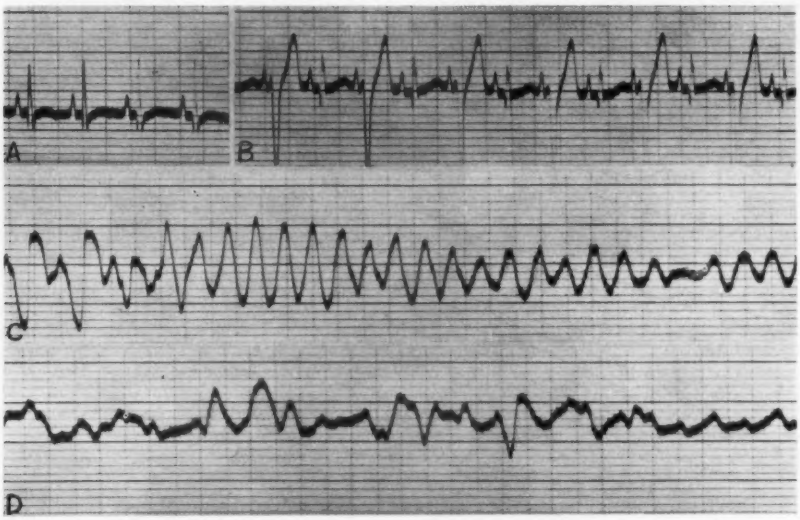


Figure 4. Electrocardiograms (lead 2) from a dog weighing 10·9 kg. before (A) and after (B, C, D) cobra venom. The first two ventricular complexes in A and B have been retouched for reproduction.

scarcely discernible. At I, four minutes later, the irregularities in the form of the ventricular complexes indicated changes in the intra-ventricular conduction path. At J, one minute later, there was a further change in the type of ventricular complex which persisted till death. The last record, K, taken from the dying dog, 80 minutes after the injection, showed great slowing of ventricular beat and diminished voltage of the ventricular complexes.

In other experiments there were additional changes and terminal events of a different kind. In one dog, block did not occur. Fifteen minutes after the injection of 2·0 mg. per kg. of venom, tachycardia, probably of ventricular origin, commenced, exhibited various types of aberrant ventricular complexes and terminated within five minutes in ventricular fibrillation. In another experiment from which

Fig. 4 is taken, the dog had received 1·5 mg. per kg. of venom. (A) is the record immediately before the injection of venom. At (B), 8 minutes later, coupled beats were observed. The rhythm returned to normal and after a further five minutes the heart went into 2 : 1 block, which rapidly developed to complete dissociation. This persisted, showing many aberrant types of ventricular complexes for nearly three hours, during which the blood-pressure was maintained at a high level (120/130 mm. Hg). The intravenous injection of a second dose of venom (1 mg. per kg.) was followed immediately by ventricular tachycardia, passing over rapidly into fibrillation. These terminal events are shown in Fig. 4 C and D taken within 2½ minutes and a few seconds respectively of the complete cessation of ventricular activity.

Post Mortem.

In dogs which died after injections of cobra venom there were subendocardial haemorrhages in the left, but not in the right, ventricle. We have not examined the heart histologically and therefore are not able to correlate lesions in the conducting tissues with the changes in conduction revealed in the electrocardiograms.

EFFECT ON PULMONARY CIRCULATION AND LUNG.

Cobra venom even in large doses (1–2 mg. per kg.) causes no obstruction in the pulmonary circulation, the pressure in the pulmonary artery remaining unaltered (Fig. 1, A). The pulmonary vessels of the dog differ from those of the cat in their reactivity to this venom. In the cat the lung vessels are peculiarly sensitive and the immediate fall of arterial blood pressure which the venom produces is accounted for by obstruction in the pulmonary circulation (Feldberg and Kellaway, 1937b). The pulmonary vessels of the dog are also relatively insensitive to histamine. The fall of arterial pressure following intravenous injection of 0·027 mg. per kg. of histamine in Fig. 1, B was associated with only a trivial rise in pressure in the pulmonary artery.

The intense haemorrhagic oedema produced by this venom in the cat's lung does not occur in the dog. Apart from patchy areas of haemorrhage, particularly in the dependent portion of the diaphragmatic lobes, there is little change after the injection of venom. The lungs do not become fleshy or oedematous. If the left lung and the intermediate lobe are removed before the injection of venom their weight is approximately equal to that of the right lung, indicating that no significant accumulation of fluid has occurred in the latter.

EFFECT ON PERIPHERAL VESSELS.

We have studied the effects of the arterial injection of small doses of venom into a hind limb and into a loop of ileum. These injections usually caused only local vascular effects without change in the arterial blood pressure; but in a few experiments an increase in systemic pressure was observed of 80–120 mm. Hg

lasting for nearly thirty minutes. Such large pressor responses cannot be explained by local vasoconstriction in the limb or bowel nor by the entry of venom into the general circulation, since the dose of venom used (1·0 mg.) never caused a pressor response by intravenous injection. It seems likely therefore that the venom acts by stimulating sensory nerve endings in the limb or bowel and that the rise occurs by reflex vasoconstriction. This effect will be described in detail elsewhere. We shall describe here only those experiments in which the systemic pressure was not altered by the arterial injection of venom and in which the changes of venous outflow were caused by the local vascular reactions.

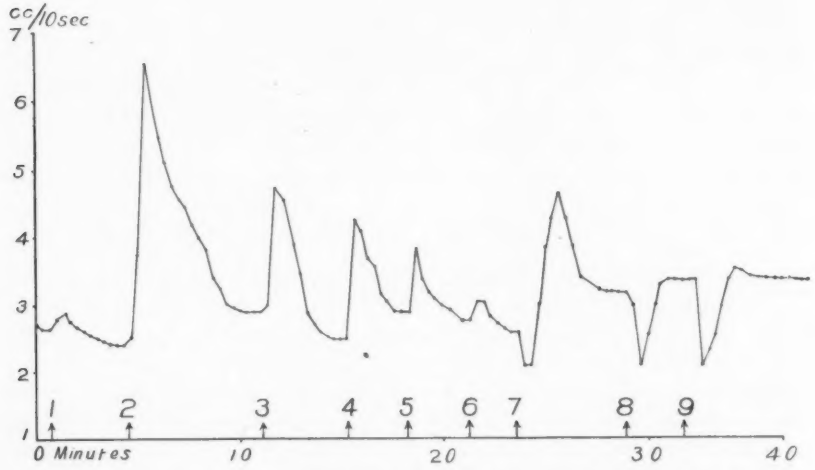


Figure 5. Venous outflow from the right hind limb of a dog weighing 10·8 kg. Ordinates, outflow in c.c. per 10 sec. Abscissae, time in minutes. At 1, saline, at 2, 3, 4, 5 and 6, 0·1 mg., at 7 and 8, 1 mg., at 9, 2 mg. of cobra venom intra-arterially. Volume of injection 0·2 c.c.

Hind Limb.

We can confirm the observation of Gautrelet, Halpern and Corteggiani (1934) that cobra venom has a strong dilator effect on the vessels of the hind limb of the dog. We found further that with successive doses of venom the vessels become less sensitive to the vasodilator action and eventually, if the dose is sufficiently large, exhibit vasoconstriction. These changes are in accord with the depressor action on the arterial blood pressure of a first injection of venom and the pressor action of a second large dose. The effects of successive arterial injections of 0·1 mg. of venom on the venous outflow of the limb are shown in Fig. 5. The arterial blood pressure remained practically constant throughout (110 to 100 mm. Hg). The dilatation became less with each injection and by the fifth (at 6) was no larger than that produced by saline (at 1). A large dose of venom (1 mg.) produced

now a sharp constriction followed by dilatation (7). With subsequent injections constriction became more, and dilatation less, pronounced (8 and 9).

Intestine.

In contrast to the behaviour of the limb vessels those of the intestine responded to the first injection of venom by vasoconstriction followed by slight and evanescent dilatation. The venous outflow from a loop of ileum diminished immediately after the arterial injection of 0·2 mg. of venom and was afterwards slightly increased for a few seconds. Repeated arterial injections of venom gave increasing constrictor responses without after-dilatation. This constriction is responsible for the initial rise in arterial blood pressure and the initial fall of pressure in the portal vein which may precede the main changes of pressure caused by the intravenous injection of a first large dose of venom.

In addition to the effects on the venous outflow, the venom had a more delayed action causing congestion and haemorrhages in the mucous membrane. These changes, which were only observed when sufficient time had elapsed between an intravenous injection (1–2 mg. per kg. of venom) and the death of the animal, were most pronounced in the duodenum and upper part of the jejunum; they were progressively less evident in the lower parts of the small intestine. The last few centimetres of the ileum and the large intestine were unaffected, even in those instances in which there was fresh blood in the faeces. The mucous membrane of the stomach was not affected or showed only congestion. The contrast between the pyloric mucous membrane and that of the duodenum was sharply defined. In those experiments in which the haemorrhages were most extensive the whole of the mucosa of the duodenum and upper part of the jejunum was dark purple and swollen; when the haemorrhages were not so extensive they appeared, in this region, as more or less parallel longitudinal stripes upon a somewhat congested but otherwise normal mucosa. A typical experiment is illustrated in Fig. 12c on page 479 of the following paper. With smaller doses of venom, the lesions in the mucosa were confined to the duodenum and upper part of the jejunum, or to the duodenum only, and the mucous membrane was sometimes congested without obvious haemorrhages.

In a dog desensitized by progressively increasing doses of venom, given intravenously at intervals of 5 to 10 minutes, the haemorrhages in the duodenum were minimal, despite the final administration, in a single dose, of 1 mg. per kg. of venom, which caused death by cardiac failure after about half an hour. It appears therefore that it is possible to desensitize the animal, not only against the peripheral vasodilatation, but also against the damage to the vessel walls which results in haemorrhages.

In some dogs haemorrhagic changes in the mucous membrane of the alimentary tract, similar in distribution and intensity to those caused by cobra venom, were also observed after the intravenous injection of histamine (0·4 to 2 mg. per kg.). See Fig. 12b on page 479 of the following paper.

EFFECT ON LIVER, PORTAL CIRCULATION AND LYMPH FLOW.

In the dog, cobra venom causes constriction of the hepatic veins and injury to the liver cells. The liver is engorged as in anaphylaxis and peptone shock. The changes in portal pressure and lymph flow, which occur after the injection of venom, are in part the outcome of these actions on the liver.

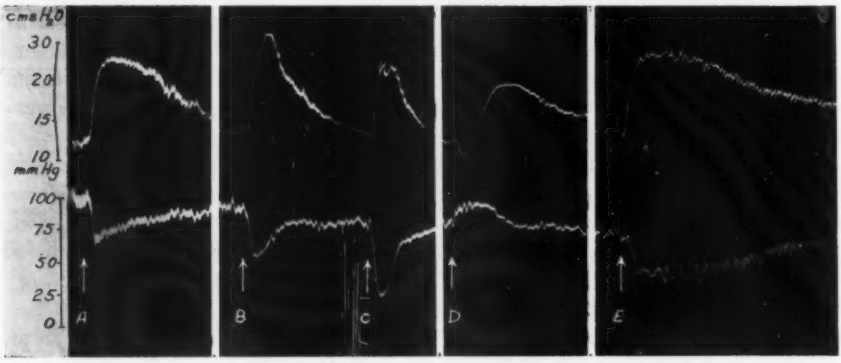


Figure 6. Pressure in the portal vein (upper tracing) and in the left carotid artery (lower tracing) from a dog weighing 8 kg. At A and E, 1·0 mg. per kg. of cobra venom injected intraportally. At D, same dose of venom intravenously. At B and C, 0·02 mg. per kg. of histamine hydrochloride injected at B intraportally and C intravenously. Time in half minutes.

Effect on Pressure in the Portal Vein.

The injection of 1 mg. per kg. of cobra venom into a femoral vein usually produces a rise preceded by a slight transient fall of portal pressure. This is illustrated in the experiment of Fig. 1, A. The initial fall of portal pressure occurred simultaneously with the rise of arterial blood pressure, but the following fall in systemic pressure started a little earlier than the rise in portal pressure. After a second injection of the same dose the initial fall in portal pressure was enhanced (Fig. 1, C). The intravenous injection of 0·25 mg. per kg. of venom occasionally caused a rise in portal pressure of a few cm. of water, but 0·1 mg. per kg. was always ineffective.

The initial fall in portal pressure is not the result of changes in the liver but is accounted for by constriction of the splanchnic vessels. This conclusion is based on the following facts:

1. Cobra venom constricts the vessels of the intestine (see page 449).

2. The initial fall in portal pressure is absent when the venom is injected into a mesenteric vein (Figs. 6, A and 8, B).

3. A small fall in portal pressure may be the sole result when the venom is injected into the superior mesenteric artery. This is shown in Fig. 7 which was obtained in the following way. The stomach, spleen and large intestine of a dog were removed, without interfering with the arterial supply to the liver; the portal pressure was recorded from a cannula tied into the splenic vein and 0·07 mg. per kg. of venom was injected into the central stump of the first branch of the superior mesenteric artery. The venom thus had to go through the small intestine before reaching the liver. Fig. 7 shows the response to the second dose of venom. A previous similar injection had caused a fall in portal pressure followed by a slight rise, which may have resulted from obstruction in the liver or from after-dilatation of the splanchnic vessels (see page 449).

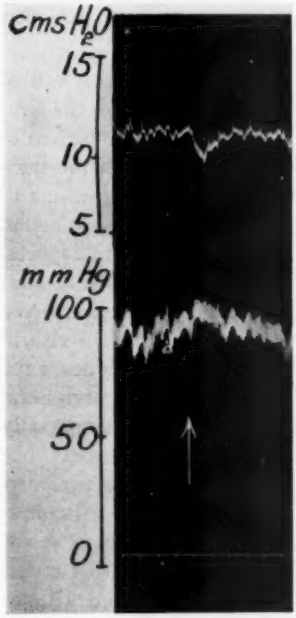


Figure 7. Pressure in the portal vein (upper tracing) and in the left carotid artery (lower tracing) of a dog weighing 14·4 kg. Stomach, spleen and large intestine removed. At the arrow (↑) 0·07 mg. per kg. of cobra venom injected into the superior mesenteric artery. Time in half minutes.

The rise in portal pressure after the intravenous injection of venom is mainly the result of obstruction in the hepatic circulation. This conclusion is supported by the results of intraportal injections of venom, after which the rise in portal pressure started earlier, was not preceded by a fall and could be obtained with a dose of venom (0·1 mg. per kg.) which was ineffective intravenously. An after-dilatation in the splanchnic area may contribute to the rise in portal pressure observed after a first intravenous injection of venom.

In a few experiments a single intraportal injection of 0·5 mg. per kg. of venom was sufficient to desensitize the hepatic vessels, a second injection producing no rise in portal pressure. In most experiments two or three successive intraportal injections of 0·5–1 mg. per kg. were necessary to diminish the response in the portal pressure.

The Rôle of the Liver in the Depressor Action of the Venom.

In different forms of circulatory shock in dogs the part played by the liver varies greatly in importance. In histamine shock the liver plays only an insignificant rôle as far as the fall of blood pressure is concerned. In anaphylaxis the circulatory failure is accounted for almost entirely by the action of the antigen on the sensitized liver (Manwaring 1911, Manwaring *et al*, 1925). Cobra venom

occupies an intermediate position resembling in this respect peptone (Feldberg, Schilf and Zernik, 1928).

The dilatation observed in the hind limb after arterial injections of venom (page 448) and the occurrence of a depressor effect after exclusion of the liver from circulation, show that the changes brought about in the liver are not solely responsible for the fall in arterial blood pressure. To exclude the liver, the dogs were eviscerated and the hepatic artery tied. In such experiments intravenous injections of 0·5 to 2·0 mg. per kg. of venom invariably resulted in a fall of systemic blood-pressure. On the other hand the effects of the venom on the liver may cause a fall in arterial blood pressure. This has been shown by comparison of the results of intravenous with those of intraportal injections. When the peripheral vasodilator mechanism has been desensitized by preceding injections of small doses of venom, doses which are ineffective or which have a pressor action when injected intravenously, cause a fall of systemic blood pressure by intraportal injection. The dog of Fig. 8 had been given an intraportal and an intravenous injection of 0·075 mg. per kg. of venom. At A, 0·5 mg. per kg. of venom given intravenously was without effect; given intraportally at B, it caused a rise in portal pressure, followed by a fall in systemic pressure. With larger doses the results may be complicated by the constrictor effect of the venom on the peripheral vessels (see page 448). In Fig. 6, 1 mg. per kg. of venom was injected intraportally at A and E, causing at both a rise in portal and a fall in systemic pressure. The same dose injected into the femoral vein at D caused a rise in the arterial pressure. The portal pressure after an initial fall also rose but not so high as after the preceding and subsequent intraportal injections. It is conceivable that the effect of the venom on the liver, if it had not been counteracted by vasoconstriction at the periphery, would have caused a fall of systemic blood pressure at D. For instance in the experiment of Fig. 1, a second intravenous injection of 1 mg. per kg. of venom at C caused a rise in arterial pressure followed by a slight fall which, since it was associated with a rise in portal pressure, was probably dependent on changes in the liver.

For comparison the effects of intravenous and intraportal injections of 0·02 mg. per kg. of histamine are shown in Fig. 6, B and C. It will be seen that in contrast to the effects of venom (at A, D and E) the depressor action of histamine is stronger when it is injected intravenously, despite the larger rise in portal pressure after the intraportal injection.

Obstruction in the hepatic circulation, if it is located at the hepatic veins, will produce a fall in systemic pressure (Mautner and Pick, 1915; Simonds and Brandes, 1925). Such an effect, however, is not produced by occlusion of the portal vein for a few minutes (Simonds and Brandes, 1925; Feldberg, Schilf and Zernik, 1928). Cobra venom must therefore constrict the hepatic veins and that part of the depressor action, which is dependent upon this effect, does not result directly from obstruction but from accumulation of blood in the liver. An accen-

tuation of this effect may be brought about by the injurious action of the venom on the liver cells, causing additional accumulation of fluid in this organ. There is yet another way in which the effect of cobra venom on the liver may lead to a fall in systemic pressure. In dogs, histamine may be liberated in the liver in such amounts that part of it may reach the blood stream in effective concentrations.

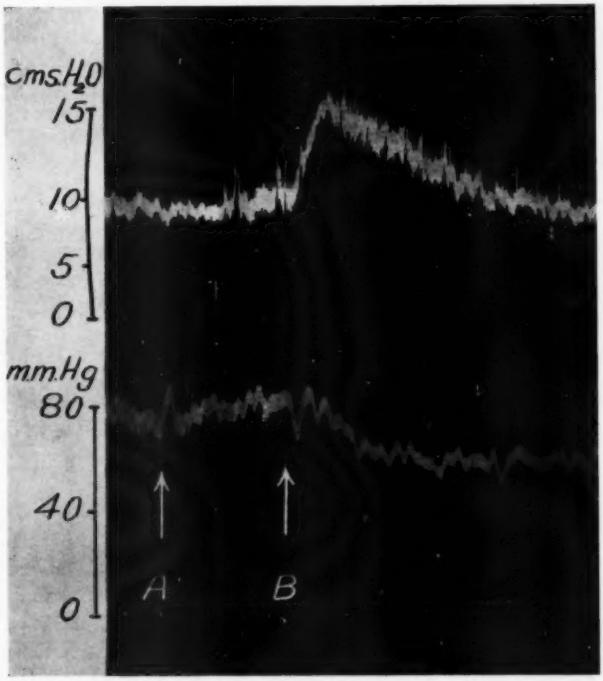


Figure 8. Pressure in the portal vein (upper tracing) and in the left carotid artery (lower tracing) from a dog weighing 7·4 kg. Injections of 0·5 mg. per kg. of cobra venom at A, intravenously, at B, intraportally. Time in half minutes.

Effect on Lymph flow.

The venom produces an increase in lymph flow which starts about one to three minutes after an intravenous or intraportal injection of 0·2 to 1 mg. per kg. of venom. The flow may increase five or six times within ten to fifteen minutes and then decrease again. The increase takes place during the fall in systemic pressure and reaches its maximum often before the blood pressure has fully recovered. This increased lymph flow is probably mainly the result of constriction of the hepatic veins and injury to the liver cells.

The lymph before injection of venom was sometimes clear, but more often turbid owing to the presence of fat. The fat content increased after the injection of venom. When the samples were collected during short periods the increase in fat was found not to occur for 3 or 4 minutes after the injection, and in the first three-minute sample there was sometimes an actual decrease in turbidity. Lymph collected during the height of the increase in flow was often richest in fat. In the following samples the fat content rapidly decreased.

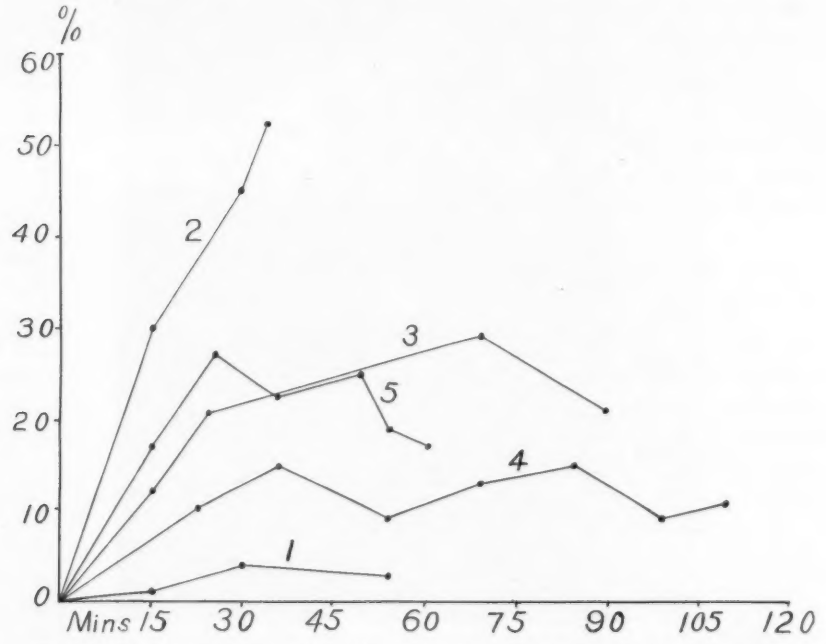


Figure 9. Increase of haemoglobin after the injection of cobra venom in normal dogs (1 and 2) and after removal of the spleen (3, 4, and 5). Ordinates percentage increase in haemoglobin values; Abscissae: time in minutes (for details see text).

An invariable feature, after both intravenous and intraportal injection, was the appearance of blood corpuscles and pigment from haemolysed red blood cells in the lymph. After the injection of 0·5 mg. per kg. of venom or more, blood appeared in the lymph in from 5 to 25 minutes, with smaller doses of venom (0·25 mg. per kg.) given intraportally, it was not observed until 40–45 minutes after injection. The blood content of the lymph became progressively greater and samples collected 2 to 4 hours after the injection gave haemoglobin values of 2 to 5 p.c. as compared with the average value of 74 p.c. haemoglobin for dogs' blood estimated by Sahli's method.

In a few dogs we have injected histamine (0·5 mg. per kg.) intraportally or intravenously. There was an increase in lymph flow which started a few seconds after the injection and reached its maximum during the first five minutes. Some thirty minutes later blood appeared in the lymph after intraportal, but not after intravenous, injections. After intravenous injections the lymph became clear and assumed an increasingly yellowish tint, giving positive Gmelin's and Van den Bergh's tests for bile pigments. Even when there were subsequently shown to be extensive haemorrhages in the duodenum and jejunum the lymph remained free from blood. This suggests that the appearance of blood in the lymph is dependent on changes in the liver.

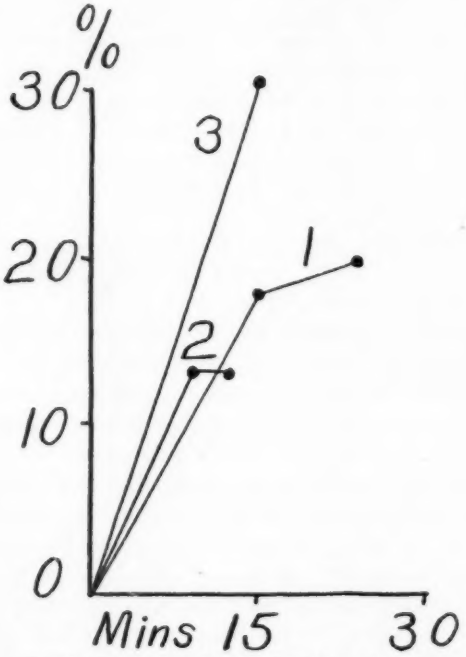


Figure 10. Increase of haemoglobin after the injection of cobra venom (1 = 1·6 mg. per kg; 2 and 3 = 2 mg. per kg.) in dogs which had been eviscerated. Ordinates and abscissae as in Fig. 9 (for details see text).

FLUID LOSS FROM CIRCULATION.

The depressor action of cobra venom, like that of histamine, is attended by loss of fluid from the circulation. This was shown by the increase of the haemoglobin concentration of the blood after intravenous injection of 1·5 to 2 mg. per kg. of venom.

Our results are graphed in Figs. 9 and 10. The abscissae give the time in minutes, after the injection of venom, the ordinates the increase per cent. of the haemoglobin values. Graphs 1 and 2 (Fig. 9) show the effect of 1 and 2 mg. per kg. respectively. The increase in haemoglobin after 1 mg. was only 4 p.c. After 2 mg. the haemoglobin value increased from 67 to 87 (i.e. 30 p.c.) within the first fifteen minutes, and to 102 (i.e. 52 p.c.) within the next twenty minutes. The last sample of blood was taken shortly before death. *Post mortem* the spleen was found to be contracted and it seemed likely that its contraction might have contributed to the increase in haemoglobin, particularly in the last two samples. This proved to be the case. But in dogs in which the spleen had been removed an increase of between 15 and 30 p.c. in the haemoglobin concentration was still observed after the injection of 1·5, 1·8 and 2 mg. per kg. of venom (Fig. 9, graphs 3, 4 and 5). This increase took place rapidly and did not progress after the first hour.

We have described a similar blood corpuscle concentration after the injection of this venom in cats, and have shown that in this species oedema of the lung accounts for part of the fluid loss. In dogs oedema of the lungs does not occur and fluid accumulation in them cannot be held responsible. The observed congestion of the liver in the dog contributes little to the effect, since its exclusion from circulation by evisceration and ligation of the hepatic artery did not prevent the occurrence of concentration. Under these conditions the animals did not survive the injection (1·6 to 2 mg. per kg.) for more than 25 minutes, but within this time the haemoglobin concentration increased from between 13 to 31 p.c. (Fig. 10, graphs 1, 2 and 3). The increase in haemoglobin must therefore be attributed to fluid loss from the circulation brought about by general increase in the permeability of the capillaries.

DISCUSSION.

In the dog the intravenous injection of the venom of the Indian cobra causes a fall of systemic blood pressure. We have recently shown that this venom also has a depressor action in the cat, but analysis in the two species of these effects has shown that they result from different causes. In the cat, the fall of blood pressure was shown to be caused by constriction of the pulmonary vessels. In the dog, the lung vessels are not constricted by the venom and the fall of arterial pressure results from vasodilatation in the parietes and is accentuated by constriction of the hepatic veins. In both species, the depressor effect is accompanied by loss of fluid from circulation but, whereas, in the cat, part of the fluid loss is accounted for by oedema of the lungs, in the dog, oedema of the lungs is absent, and the whole of the fluid loss must be attributed to changes in the permeability of the capillaries of the general circulation. In the cat, we were unable to demonstrate an immediate vasodilator effect of the venom in the hind limb, the vessels of the limb reacting like those in the splanchnic region of the dog; furthermore, the effects of the

venom upon the liver in the cat do not contribute either to the fall of blood pressure or to the loss of circulating fluid.

These differences in the circulatory effects of cobra venom observed in the dog and cat also occur with other cell-injurious agents, and, to a lesser degree, with histamine itself. Anaphylactic shock in the cat is extremely difficult to produce, but in the dog the circulatory symptoms are dependent upon changes in the liver. Peptone has a strong hepatic action in the dog which contributes to its depressor effect, whereas in the cat the fall of systemic blood-pressure is partly the result of obstruction in the pulmonary circulation and constriction of the hepatic veins does not occur. The early depressor effect of staphylococcal toxin is the result in the cat of pulmonary constriction and, in the dog, of changes in the liver. Toxic action on the heart plays an important part in the fatal termination, in both species (Kellaway, Burnet and Williams, 1930; Feldberg, Kellaway and Keogh). In histamine shock the main effect in both species is peripheral vasodilatation but large doses constrict the pulmonary vessels in the cat and the hepatic veins in the dog and affect the heart in both species.

Despite the differences between the circulatory effects of cobra venom in the cat and in the dog, there are many points of resemblance. In both animals, the venom has a powerful toxic action upon the heart which may determine the fatal issue after large doses. In both, the venom causes increase in the permeability of the capillaries. In both, a first dose of the venom has a desensitizing action and a second large dose causes a rise instead of a fall of blood-pressure. A more complete analysis of the effects of the venom upon various parts of the "vascular tree" might show that the reactions of the peripheral vessels to the venom in the two species have more in common than is apparent from the observed changes in the venous outflow of the hind limb. It appears that the capillaries are dilated in both species by the venom. In both, an increase in their permeability is indicated by the fluid loss from circulation and we may reasonably infer that this change is associated with dilatation. The measurement of the venous outflow does not differentiate between simultaneously occurring events in the arterioles, capillaries and veins. Although we observed a decrease in the venous outflow from the cat's limb and an increase in the outflow from the dog's limb after arterial injections of small doses of venom, the capillaries may have been dilated in both and the difference accounted for by reactions on the arterial side of the vascular tree, which is constricted in the cat and dilated in the dog. Even in the dog large doses of venom decrease the venous outflow from the limb. Differences in the reactions of the arterial side of the vascular tree are therefore in part quantitative rather than qualitative. This interpretation of the vascular effects of the venom has much in common with that given for histamine, which constricts the arterioles in the cat and dilates them in the dog, but causes dilatation of the capillaries in both species (Dale and Richards, 1918).

In the interpretation of the vascular effects there is another possibility that

must be kept in mind. The constrictor actions of this venom may be caused by the presence of an active principle distinct from that which causes vasodilatation. The results of experiments in which the animals were desensitized against the vasodilator action of the venom and responded by constriction to large doses, offer a hint that this may be the case. The constrictor effect on the lung vessels in the cat and part of the constriction in the hepatic circulation in the dog may be caused by such a hypothetical constrictor principle and the impairment of conduction observed in the heart, may result from constriction by this hypothetical constrictor principle in the coronary circulaion. An analogy is afforded by observations (Feldberg and Kellaway, 1937d) in the dog and cat on the circulatory effects of bee venom, which in its raw state contains a constrictor principle the concentrations of which is greatly diminished by repeated precipitation with alcohol.

The vasoconstriction observed in the ileum and hind limb after the arterial injection of venom suffices to explain the initial transient pressor response preceding the main fall of systemic pressure caused by the intravenous injection of a first large dose of venom, as well as the more pronounced pressor effect of a second dose. There is, however, the possibility that the venom stimulates sensory nerve endings at the periphery and by reflex action causes a rise in systemic pressure. This mechanism may be responsible for the pressor effects described by other workers (Chopra and Iswariah, 1931).

Our experiments have been directed towards finding out what part of the circulatory effects of the venom may be attributed to the liberation of histamine. We have already drawn attention to the similarity between the vascular effects of venom and those of histamine in the cat and dog. The characteristic distribution of the haemorrhages in the mucosa of the intestinal tract after the injection of venom may be explained by the liberation of histamine in these areas, since the intravenous injection of histamine causes similar lesions with a similar distribution. Other cell-injurious agents (histamine-free peptone and bee venom) also produce haemorrhages of the same character and distribution (Feldberg and Kellaway, 1937d). An increase in the pressure in the portal vein and increased lymph flow are observed, after venom, as well as after histamine. The appearance of blood corpuscles in the lymph seemed at first sight to be the result of a direct cell-injurious action of the venom, because it was not observed after intravenous injection of histamine. Intraportal injections of histamine were, however, followed by the appearance of blood in the lymph and it therefore seemed probable that strong constriction in the hepatic veins is an adequate cause for diapedesis. The direct cell injurious action on the liver is indicated by the fact that the liver is more congested and firmer after venom than after the injection of histamine. Even the toxic effects of the venom on the heart show resemblances to the effects of histamine. Interference with the conducting mechanism (Hashimoto, 1925) and haemorrhages in the heart (Sieburg, 1914) have been observed in dogs after histamine, though the changes were not so pronounced as those found after venom.

Cobra venom shares this resemblance with many other cardiac poisons, and this fact indicates the possibility that a common intermediary, like histamine, may be responsible.

In 1915 Mautner and Pick suggested that circulatory shock caused by different substances was the outcome of obstruction in the pulmonary and portal circulations and spoke of the lung in cats and of the liver in dogs as "shock organs". This conception failed to recognize the importance of peripheral vasodilatation, which in some forms of shock, as with histamine, is the determining factor, in others as with peptone, contributes greatly to the circulatory effects and which in yet others may be trivial, as in anaphylaxis. The conception of "shock organs" may be enlarged to include those morbid changes, which occur after toxins and venoms and which appear to be, in part, the effect of liberated histamine, and, in part, of cell injury itself. If the conception of "shock organs" embraces the idea that different organs in different animals are particularly sensitive to cell injury, then we may say that in the dog the liver, the mucosa of the upper part of the intestine and the heart, and in the cat, the lung and the heart, appear to play this part, and the actions of various cell-injurious agents may differ in so far as they are more or less confined to these organs.

SUMMARY.

1. In the dog the intravenous injection of a first dose of cobra venom (0·2–2 mg. per kg.) causes a fall of arterial blood-pressure. After large doses (1 mg. or more per kg.) the fall is preceded by a small pressor effect. A second injection of venom is either ineffective (small doses) or causes a rise in arterial pressure (large doses).

2. The initial fall of arterial blood-pressure is not caused by failure of the heart as indicated by the concomitant fall in venous pressure. At variable times after the injection of large doses of venom (1·5 to 2 mg. per kg.) cardiac failure with a rise in venous pressure and a final fall in systemic pressure occurs. This is preceded by impairment of conduction in the heart as manifested by heart block and abnormal ventricular rhythms. *Post mortem* there are subendocardial haemorrhages in the left ventricle.

3. The fall of arterial blood pressure is not caused by obstruction in the pulmonary circulation. In dogs cobra venom does not cause a rise of pressure in the pulmonary artery nor oedema of the lungs.

4. The initial fall of arterial pressure is dependent upon vasodilatation as demonstrated in the vessels of the hind limb. The vessels of the splanchnic area first contract and then undergo transient dilatation. The contraction of the splanchnic vessels may account for the immediate rise in systemic pressure after intravenous injections of large doses of venom. *Post mortem* there are engorgement and haemorrhages in the mucosa of the duodenum, contrasting sharply with

the normal or slightly congested mucosa of the stomach. The haemorrhages decrease progressively in the jejunum and ileum. The mucosa of the large intestine is only slightly congested. Similar changes have been observed after the injection of *histamine*.

5. The initial fall in systemic pressure is associated with a rise of pressure in the portal vein. In dogs cobra venom causes constriction of the hepatic veins and injury to the liver cells which contributes to the fall of systemic pressure. The lymph flow is increased and contains blood corpuscles and haemoglobin.

6. The fall in systemic pressure is associated with increased haemoglobin concentration of the blood as the result of fluid loss from circulation brought about by changes in the permeability of the capillaries.

ACKNOWLEDGMENTS.

Dr. Hume Turnbull has gone through our electrocardiographic records, which were taken for us by Mr. E. Hughes. The haemoglobin estimations were done for us in Dr. Gardner's department. We should like to make grateful acknowledgment of this assistance.

REFERENCES.

Chopra, R. N. and Iswariah, V. (1931): Indian J. Med. Res., 18, p. 1113.
Dale, H. H. and Richards, A. N. (1918): J. Physiol., 52, p. 110.
Feldberg, W. and Kellaway, C. H. (1937a): J. Physiol., 90, p. 257.
Feldberg, W. and Kellaway, C. H. (1937b): Austral. J. exp. Biol., 15, p. 159.
Feldberg, W. and Kellaway, C. H. (1937c): Austral. J. exp. Biol., 15, p. 81.
Feldberg, W. and Kellaway, C. H. (1937d): Austral. J. exp. Biol., 15, p. 461.
Feldberg, W., Kellaway, C. H. and Keogh, E. V. (unpublished experiments).
Feldberg, W. and Schilf, E. (1930): "Histamin". Springer, Berlin, p. 229.
Feldberg, W., Schilf, E. and Zernik, H. (1928): Pflügers Arch., 220, p. 738.
Gautrelet, G. and Halpern, N. (1934): C.R. Soc. Biol., Paris, 115, p. 942.
Gautrelet, G., Halpern, N. and Corteggiani, E. (1934): Arch. int. Physiol., 38, p. 293.
Hashimoto, H. (1925): Arch. intern. Med., 35, p. 609.
Kellaway, C. H., Burnet, F. M. and Williams, F. E. (1930): J. Path. Bact., Lond., 33, p. 889.
Laignel-Lavastine, Würmser, L. and Koressios, N. T. (1934): Bull. Soc. méd. Hôp., Paris, p. 494.
Manwaring, W. H. (1911): Z. Immunitätsforsch., 8, p. 1.
Manwaring, W. H., Hosepian, V. M., Enright, J. R. and Porter, D. F. (1925): J. Immunol., 10. p. 567.
Manwaring, W. H., Hosepian, V. M., O'Neill, F. F. and Moy, H. B. (1925): J. Immunol., 10, p. 575.
Mautner, H. and Pick, E. P. (1915): Münch. med. Wschr., p. 1141.
Simonds, G. P. and Brandes, W. W. (1925): Amer. J. Physiol., 72, p. 320.
Vernes, A. and Koressios, N. T. (1934): Bull. Médical, 20 Janvier 1934, quoted after Laignel-Lavastine, Würmser and Koressios, 1934.

LIBERATION OF HISTAMINE AND ITS RÔLE IN THE SYMPTOMATOLOGY OF BEE VENOM POISONING

by

W. FELDBERG AND C. H. KELLAWAY

(From the Walter and Eliza Hall Institute, Melbourne).

(Submitted for publication 16th October, 1937.)

The effects of bee venom have often been compared with those produced by the venoms of certain snakes (Langer, 1897; Morgenroth and Carpi, 1906; Essex, Markowitz and Mann, 1930; Tetsch and Wolff, 1936). In experiments on perfused lungs we have recently shown (1937a) that some snake venoms cause the liberation of histamine and that this mechanism may account for many of their actions on smooth muscle and on the circulation. In the present experiments we have sought to ascertain whether a similar mechanism is concerned in the action of bee venom.

The effects of this venom in animals (see Phisalix, 1922) indicate that in many details they resemble those of histamine. Essex, Markowitz and Mann (1930) have stressed this similarity. They write: "The venom of the bee may be described as a violent endothelial poison and a marked stimulant of smooth muscle. It is comparable in these respects to histamine." Recently Nagamitu (1935) and Reinert (1936) have shown that histamine is present in bee venom. Reinert succeeded in isolating from 1 gm. of crude venom 10 mg. of histamine. The presence of histamine in such high concentration in bee venom detracts from the value of much of the early work. Attempts have been made to distinguish between the effects of histamine and those of other active constituents of the venom. According to Nagamitu histamine is responsible for wheal formation when the venom is punctured into the human skin, but the haemolytic action of the venom is caused by another active principle. Reinert and Tetsch and Wolff state that the action of the venom on the gut is the effect of two different stimulating principles, one of which is histamine.

METHODS.

The experiments were carried out on guinea-pigs, cats and dogs. The liberation of histamine was studied in perfusion experiments on lungs from guinea-pigs and dogs, and on livers from dogs. The arrangement for lung perfusion was that described for guinea-pig's and cat's lungs in previous experiments (1937a), simplified as described by Feldberg and Keogh (1937). The dog's liver was perfused through the portal vein with warm Tyrode solution, only part of the organ (35 to 90 gm. in weight) being used. The rate of perfusion was between 1 and 3 c.c. per minute.

Under morphine and chloroform anaesthesia the abdomen was opened, the hepatic artery tied, a cannula was inserted into the portal vein and the perfusion was commenced. The gastro-duodenal vein was tied since it entered the portal vein cranial to the cannula. The thorax was opened by cutting through the diaphragm and the inferior vena cava was divided. The left and central lobes of the liver with the gall bladder were tied off and excised, and the remainder of the liver (the right lateral lobe with the *processus caudatus* and the *processus papillaris*) was freed from the body and placed in a tray of solid paraffin from which the outflow was collected. The lymphatics in the *porta hepatis* were opened. The *processus papillaris* was tied off and excised, part of it being used to make saline extract. The method of extraction was that used in the lung perfusion experiments (1937a). In those experiments in which both the liver and part of the lung were perfused the arrangements for the liver perfusion were the same, but the inferior vena cava was tied close to the heart before dividing it near the diaphragm and the descending aorta was clamped in the thorax. This enabled us to start perfusion of the lung after having carried out the preparation of the liver. For the routine assay of histamine the perfusates from the lungs and livers were tested on a piece of isolated jejunum from a guinea-pig. All the values of histamine given in this and the preceding papers are expressed as histamine-dichloride and not as base.

The methods for the analysis of the circulatory effects in cats and dogs were the same as those used in the study of the venom of the Australian copperhead (1937b), and the Indian cobra (1937c, d). All experiments on cats and dogs were carried out with the vagi divided and, if not otherwise stated, under artificial ventilation. In a few experiments on cats the respiration was recorded by connecting a side branch of the tracheal cannula with a Brodie's tambour.

EXPERIMENTAL.

The guinea-pig is known to be sensitive to bee venom, which causes bronchospasm and other symptoms similar to those produced by the injection of histamine; we have therefore investigated the action of the venom in liberating histamine from the perfused lungs in this species. We have not analysed the symptomatology of poisoning by bee venom in the guinea-pig though we have studied its effects upon the isolated gut. We then examined the action of bee venom in the cat and found, in confirmation of earlier observations (see Beck, 1935) that this animal is rather insensitive. It appeared to be unsuitable for investigating by perfusion experiments the liberation of histamine by the venom. In the dog a detailed comparison of the symptoms resulting from injections of bee venom and of histamine has been made and we have observed the effect of the venom in liberating histamine from the perfused liver and lungs. In all these experiments it was necessary first to ascertain whether histamine was present in the samples of venom tested and if so in what amount.

A. HISTAMINE CONTENT OF BEE VENOM.

We have used three different batches of venom, all of which contained per c.c. the equivalent of 50 stings.[1] They were clear glycerol-water extracts, one of

[1] Preliminary experiments were made with samples of "Hermette" bee venom manufactured for therapeutic use by Mr. David G. Bull of Melbourne. Since these were insufficiently concentrated for our purpose, Mr. Bull kindly prepared for us the batches of venom used in the experiments here presented.

which had undergone only partial purification with alcohol and will be referred to as "crude venom". The other two samples had been further purified by repeated precipitation with alcohol.

When the crude venom was tested in high dilution on the isolated intestine of the guinea-pig it was found to contain a muscle-stimulant principle, which gave a rapid contraction with short latency and quick recovery and to which the gut did not become desensitized. This contraction must be attributed to histamine present in the venom since the venom caused an immediate evanescent fall of arterial blood pressure in the atropinized cat and when assayed against histamine by its depressor and gut stimulating actions the results agreed quantitatively as to the amount of histamine present. Furthermore, histamine has been isolated from raw bee venom and identified chemically (Reinert, 1936). The histamine concentration in the sample of crude venom was about 1 : 17,000.

The histamine concentration in the samples of purified venom could only be roughly assayed on the desensitized gut since they had to be used in concentrations so high, that the venom impaired the reactivity of the gut (*vide infra*), but with some preparations of guinea-pig's intestine an approximate estimate could nevertheless be obtained. Since the cat was found to be relatively insensitive to the purified venom, we were able to assay these samples more accurately on the cat's blood pressure. One contained less than 1 in 5 millions and the other about 1 in 1·5 millions of histamine. The former was used for the experiments on guinea-pigs and the latter for those on cats and dogs.

B. EXPERIMENTS ON GUINEA-PIGS.

The Isolated Gut.

The presence of large amounts of histamine in crude venom made it difficult to ascertain the effect of the other constituents of the venom. With purified venom this difficulty disappeared, since the venom was active in concentrations in which the small amount of histamine present was diluted to below its threshold of activity. Under these conditions the venom, after a latent period of 30 to 60 seconds, caused contraction of the gut, from which relaxation proceeded slowly when the Tyrode solution was changed and to which the muscle could be desensitized by repeated administration of venom. These results are illustrated in Fig. 1. At A, B, C and D are shown the first, second, third and sixth responses to $\frac{1}{200}$ of a sting. In some experiments similar results were obtained with an equivalent of $\frac{1}{2000}$ of a sting, but smaller amounts were ineffective. The venom was left in contact with the muscle for 90 seconds before changing the Tyrode solution. The sharp rises at A and B started after latent periods of 35 and 60 seconds respectively. The onset of desensitization was shown at B by increase in the latent period and diminished height of response. After washing out the venom the muscle remained contracted or even contracted further during some seconds and then relaxed gradually. The effect of the third dose of venom (at C) was small.

At D the muscle had become desensitized to doses of venom of this order though it still responded to a ten times stronger concentration at E, with a latent period of some forty seconds and a typical slow contraction. With repeated administration, the muscle became desensitized to these doses of venom; but even then desensitization was not absolute since the muscle responded to still larger doses to which it could again be desensitized. If the initial dose of venom were large (1/10 to 1 sting) the latent period was shorter, the muscle contracted more rapidly and relaxation proceeded more slowly after changing the Tyrode solution.

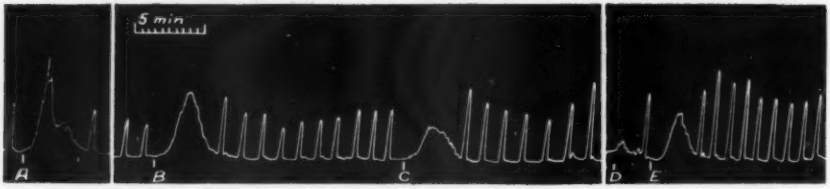


Figure 1. Isolated guinea-pig's jejunum in 3 c.c. bath of Tyrode solution. Responses to 1/200 of a sting of bee venom at A, B, C and D, and to 1/20 of a sting at E. The rapid responses of short duration are each caused by 0·04γ of histamine.

In addition to the stimulating effect of the venom the response of the gut to histamine was often characteristically altered after the administration of a small dose of venom. This is shown in Fig. 1. After each application of venom at B, C and E the muscle was first more, but later less sensitive to histamine. The condition of increased sensitivity was maximal four to six minutes, that of decreased sensitivity seven to ten minutes after the venom. The increased sensitivity could not be explained by the long pause elapsing since the preceding application of histamine, because maximal reactivity after the venom was often not obtained until the second or third dose of histamine was given (cf. histamine effects after E). Furthermore when histamine was given before the muscle had completely relaxed from the venom contraction, its effect was small. In some experiments the increased, in other the decreased, reactivity of the muscle was more pronounced.

It may be that bee venom (as well as other cell-injurious agents) not only liberates histamine (*vide infra*) but also sets free other specific or nonspecific substances which produce the observed changes in reactivity. This view is supported by the fact that, as we shall see, extracts of guinea-pig's lungs as well as samples of perfusate collected from the lung and liver after the injection of bee venom cause similar changes of the sensitivity of the gut to histamine (see Fig. 13). We have also observed after copperhead venom a period of irregularly increased reactivity of the gut to histamine (1937a).

Possibly the decreased reactivity results from damage to the muscle. If this be so it should be more pronounced after large doses of venom, which is indeed the

case. This is shown in Fig. 2. At A the response to a fourth application of a $\frac{1}{20}$th of a sting is seen. The muscle was almost completely desensitized to doses of venom of this order but responded to half a sting (at B). There was no response to a second application of this dose (at C). At this stage the response to histamine was somewhat diminished. After 5 stings (at D) it was greatly reduced and the muscle did not recover during the next half-hour.

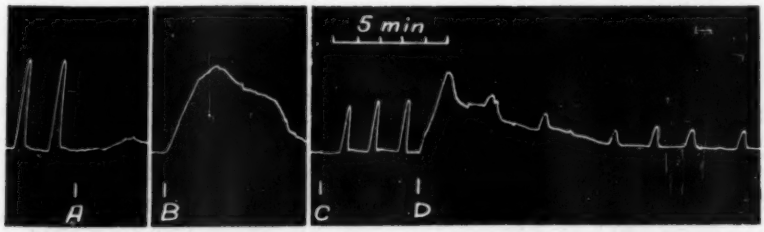


Figure 2. Isolated guinea-pig's jejunum in 3 c.c. bath. Responses to 1/20 sting (at A), to $\frac{1}{2}$ sting (at B and C) and to 5 stings (at D) of venom. The rapid responses before A and after C and D are each caused by 0·04γ of histamine.

Liberation of Histamine from the Perfused Lungs.

The injection of purified bee venom into the pulmonary artery of the perfused lung caused the appearance in the outflowing fluid of histamine, of a substance which caused a slow contraction of the isolated gut and of coagulable protein.

The fluid collected before the injection of venom was either free from activity or had only a weak stimulant action on the isolated gut. After the injection the fluid became active; the activity increased during the first 1 to $1\frac{1}{2}$ hours and then decreased again. In Fig. 3 the effect on the desensitized gut of 0·2 c.c. of a sample of perfusate collected before (at A) and of different samples collected from between 15 and 150 minutes after the injection of 1 c.c. (50 stings) of venom (at B, C, D, E and F) are shown. The samples collected after the injection caused a quick contraction which was followed, after replacing the fluid in the bath with fresh Tyrode solution, by a second slow contraction. In this experiment the slow after-contraction was particularly pronounced and an accurate assay of the principle causing rapid contraction was therefore difficult. In other experiments the after-contraction was small and did not interfere with the assay. With repeated administration of the fluid to the same piece of jejunum, its effect in causing slow contraction varied. Boiling the liquid, though it appeared to diminish the effect, did not destroy the substance responsible for it. We have not tried to ascertain the nature of this active substance, which is probably a normal cell constituent, because saline extracts of guinea-pig's lung often had a similar action. After the perfusate had been washed out from the bath, the response of the gut to histamine was often enhanced and later on diminished.

The substance which causes rapid contraction is histamine. It was identified pharmacologically, as in our corresponding experiments with snake venoms (1937a). Those samples which were active had, in the cat, a depressor action which was not abolished by atropine and when the activity of the samples was assayed against histamine on the gut and on the blood-pressure the results corresponded closely.

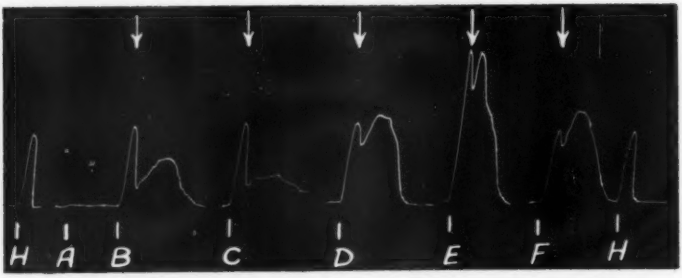


Figure 3. Isolated guinea-pig's jejunum in 3 c.c. bath. Responses to 0·04γ histamine (at H) and to 0·2 c.c. of perfusate from guinea-pig's lung collected before (at A) and 15, 30, 80, 100 and 150 minutes (at B, C, D, E and F respectively) after venom. At the arrows (↓) the Tyrode solution in bath was changed.

Any doubt as to the identification of the active substance as histamine was removed by the demonstration that it was part of the lung histamine. Normally the histamine contents of the right and left lungs do not differ by more than 20 p.c. When the right lung only was treated with venom, its histamine content assayed at the end of the experiment was found to be much lower than that of the control left lung, and the difference was accounted for by the histamine content of the perfusate (Table 1). It will be noted that in the second experiment of the table the difference between the contents of histamine in the left and right lungs exceeds 20 p.c. It is probable that the amounts estimated in the perfusate were a little too high, owing to the presence of the substance causing the slow-contraction.

TABLE 1.

Histamine Content of the Right and Left Lungs of the Guinea-pig.

Weight of guinea-pig in gm.	Histamine content of left lung in γ.	Histamine content of right lung in γ. (a), (b) and (c).	(a) in perfusate.	(b) in drainage fluid.	(c) in lung extract.	
340	41	43·0	22·1	1·7	19·2	3½ hours perfusion of right lung after 25 stings of bee venom.
392	9·8	12·4	8·8	0·4	3·2	3½ hours perfusion of right lung after 5 stings of bee venom.
370	13	14·1	9·0	—	5·1	3½ hours perfusion of right lung after 5 stings of bee venom.

In these experiments after 3½ hours' perfusion the right lung was cut away and placed in a funnel to drain. The fluid collected (drainage fluid) during 20 minutes showed in two successive samples an increasing concentration of histamine which did not, however, as in corresponding experiments with snake venoms, reach the concentration of the histamine remaining in the lungs. This latter was calculated on the assumption that the histamine was uniformly distributed in the lung tissue and the fluid remaining in the lung. The histamine concentration of the last sample of drainage fluid was two or three times less than that in the lungs instead of 50 and 100 times less as in lungs perfused but untreated with venom (Feldberg and Kellaway, 1937a). For instance in the first experiment in Table 1 the drainage fluid collected during the first five minutes (1·1 c.c.) had a histamine concentration of 1 in 1·7 millions and that collected during the next fifteen minutes (0·4 c.c.) had a histamine concentration of 1 in 350,000. The concentration of histamine calculated for the right lung (2·4 gm.) was 1 in 125,000. The high concentration of histamine in the drainage fluid indicates an increase in permeability of the cells to the histamine present in them.

The fact that histamine found in the perfusate and drainage fluid is part of the lung histamine enables us to express it as a percentage of the latter. In the three experiments of Table 1 the histamine outputs from the right lung were 55, 74 and 64 p.c. respectively. In these experiments the venom was injected in the same concentration and we do not know whether, as is the case with snake venom, higher concentrations would have been more effective in depleting the lung of its histamine store.

In Experiment 1 of Table 1, 35 mg. of coagulable protein (estimated by Esbach's reagent) were found in the perfusate collected after the injection of venom. The concentration of protein was highest in those samples which contained most histamine.

EXPERIMENTS ON CATS.

Arterial Blood Pressure.

The cat's blood pressure is rather insensitive to bee venom. In our experiments the depressor effect of small doses (1–10 stings) was accounted for by the histamine present in the venom. Large doses (25 stings or more) caused additional circulatory effects which differed according to whether crude or purified venom was used.

The injection of crude venom in a dose of 25 stings or more caused a precipitous fall of systemic blood pressure and a sharp accompanying rise of pressure in the pulmonary artery. Death occurred within a few minutes (Fig. 4a). We were able to show that the depressor action on the systemic blood pressure and the constrictor effect on the pulmonary vessels were stronger than those caused by the histamine in the venom. In the cat of Fig. 4b, the effect of 0·5 c.c. of histamine, 1 in 17,000, was compared with that of 0·5 c.c. of crude venom (25 stings), containing an equal amount of histamine. The venom caused an immediate steep

fall of systemic pressure, but instead of recovering, the blood pressure after an interval of about half a minute underwent a further fall which terminated in death. The rise of pressure in the pulmonary artery after venom was large in comparison with the trivial effect of a dose of histamine equivalent to that present in the venom. The strong obstruction in the pulmonary circulation after venom explains

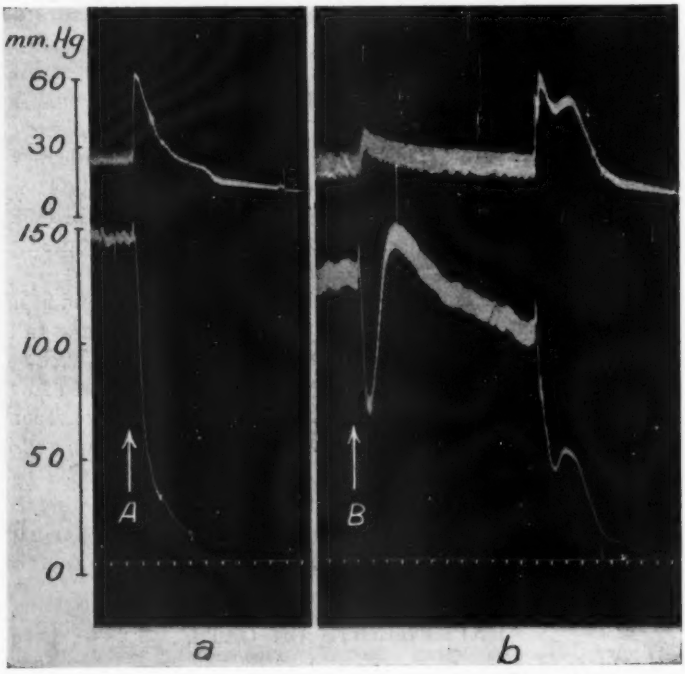


Figure 4. Blood pressure in carotid (lower tracing) and pulmonary artery (upper tracing) from a cat weighing 4 kg. under dial (a) and from a cat weighing 3 kg. under chloralose (b). At A. 100 stings of crude bee venom (1 c.c.), at B, 0·5 c.c. histamine 1: 17,000 and six minutes later 25 stings of crude bee venom (0·5 c.c.) intravenously. Time in half minutes.

the disappearance of pulsatory oscillations from the record of the systemic pressure despite their continued presence in the record from the pulmonary artery. The effects of bee venom resembled those we have observed (1937c) after the injection of the venom of the Indian cobra.

In one experiment in which recovery occurred, a second similar injection of crude bee venom again produced almost identical responses in the pressures of the carotid and pulmonary arteries. Desensitization against the constrictor action on the pulmonary vessels is therefore not readily obtained.

The purified venom which contained less histamine than the crude venom had also a weaker constrictor action on the pulmonary vessels. A rise in pressure in the pulmonary artery was only obtained after an injection of venom equivalent to 100 stings. Even this was not followed by an immediate profound fall of systemic pressure. Amounts equivalent to 25 or 50 stings generally caused a transient fall of systemic pressure corresponding to the depressor action of the histamine in the venom. In one experiment there was a rise of pressure. Generally a gradual fall of blood pressure occurred somewhat later leading to death in the course of half-an-hour to two hours. In the experiment of Fig. 5, purified

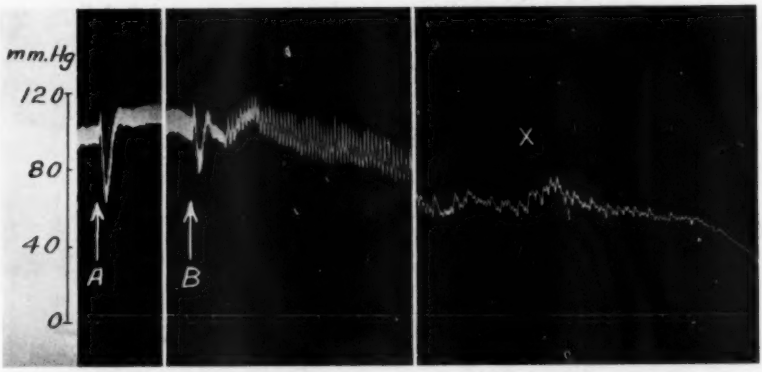


Figure 5. Tracing of carotid blood pressure from a cat weighing 2·5 kg. under dial. Artificial respiration. At A, 1 c.c. histamine 1: 1,500,000, at B, 50 stings purified bee venom (1 c.c.) intravenously. At X, artificial ventilation discontinued. Time in half minutes.

venom equivalent to 50 stings injected at B caused an immediate fall of pressure, a little less than that produced by the injection of histamine in a dose equal to that present in the venom. This transient fall was followed by a prolonged and gradual fall which was accelerated by stopping the artificial ventilation (at X) an hour after the injection. Death occurred within a few minutes. A characteristic feature of the injection of venom in artificially ventilated animals was the appearance of spontaneous respirations. The effects of these respiratory movements can be seen in the blood pressure tracing (Fig. 5). In spite of strong artificial ventilation the arterial blood was often poorly oxygenated in the later stages of the experiment.

In a few experiments we injected the venom into animals without artificial ventilation. In these the first effect was an increase in the rate and extent of respiration similar to that observed after the injection of snake venom (Kellaway and LeMessurier, 1936). The respiration became slower but was increased in

depth and the effects of respiratory movements on the arterial blood pressure were pronounced (Fig. 6). In the later stages, before death, the respiration was slowed, and was sometimes periodic or exhibited other irregularities (Fig. 6d). We have not analysed the cause of these changes in respiration which were also observed after the injection of the crude venom.

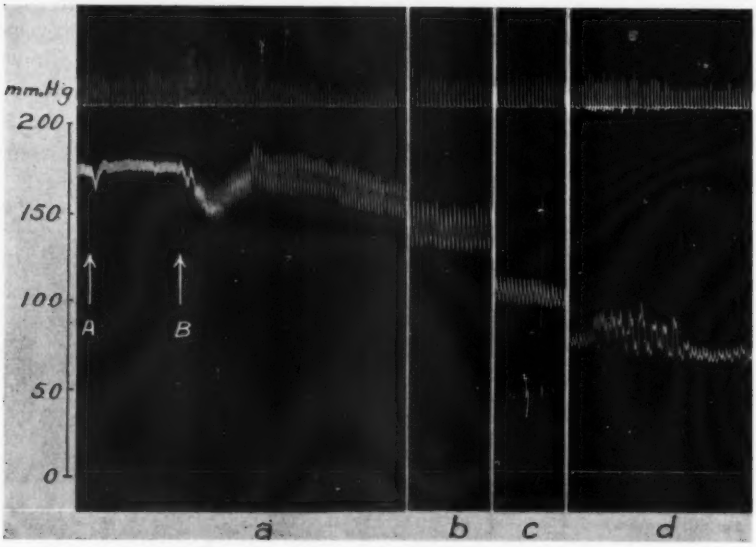


Figure 6. Tracheal record of respiration (upper tracing) and carotid blood pressure (lower tracing) from a cat weighing 3 kg., under dial. At A, 1 c.c. histamine 1: 1,500,000, at B, 50 stings purified bee venom (1 c.c.) intravenously. b, c and d are 55, 105 and 145 minutes respectively after the injection of venom. Time in half minutes.

Lungs.

The appearance of the lungs from cats dying some time after large doses of bee venom resembled those observed after cobra venom. The lungs did not collapse when the thorax was opened and artificial ventilation was discontinued. There was intense haemorrhagic oedema. In some experiments the left lung and the intermediate lobe were removed before the venom was injected. Normally their combined weight corresponds to that of the right lung, but after the injection of 50 stings of purified or crude venom, the right lung was about 50 p.c. heavier, and after the injection of 100 or 200 stings it was over 100 p.c. heavier than the combined weight of the left lung and the intermediate lobe. When these heavy lungs were allowed to drain after being excised, fluid containing only a little blood dripped from the hilus.

The lungs, after venom, were tough in texture and showed areas of haemorrhage and atelectasis. On section the appearance presented, sometimes resembled that of red hepatization, but microscopic examination showed that this was caused by intense congestion, the capillaries in the alveolar walls being stuffed with corpuscles and the alveoli full of exudate (oedema fluid). These changes in the lungs may have accounted for the failure fully to oxygenate the blood, which was often observed despite artificial ventilation.

Fluid Loss from Circulation.

These experiments had to be carried out with purified venom because the histamine content of the crude venom was so high as to cause, by itself, fluid loss from circulation. On the other hand there was not enough histamine in 1 c.c. of purified venom to cause blood corpuscle concentration. The intravenous injection of this dose of purified venom caused a rise in the haemoglobin percentage of from 15 to 24 p.c. (Table 2). As these experiments were made after removal of the spleen the increase in haemoglobin concentration must have resulted from loss of fluid from the circulation. In column 5 of Table 2 the fluid loss calculated from the increase in haemoglobin is given. Fluid accumulation in the lungs only contributed to a slight extent. In the two last experiments of the Table the left lung and the intermediate lobe were removed before the injection of venom and the fluid accumulating in the remaining lung was ascertained (column 6 of Table 2). It amounted to less than 15 p.c. of the total fluid loss. Increase in permeability of the capillary walls in general must therefore account for the main part of the fluid loss.

TABLE 2.

Effect of Purified Bee Venom (Equivalent to 50 Stings) on Haemoglobin Concentration of Cat's Blood after Removal of Spleen.

Weight in kg.	Haemoglobin. Before venom.	Haemoglobin. Maximum after venom.	Increase in p.c.	Calculated fluid loss in c.c.	Fluid loss in right lung.
2·7	69	85·5	24	26	
2·5	79·5	94·5	19	20	
3·5	78	90	15	23	3·4
4·0	76·5	91·5	20	33	4

D. EXPERIMENTS ON DOGS.

Arterial Blood Pressure.

The blood pressure of the dog, unlike that of the cat, is sensitive to bee venom. We found that an intravenous injection of purified venom, equivalent to one sting, caused a gradual fall of a few millimetres of Hg. Five to ten stings produced a profound fall, after a latent period of twenty to forty seconds; the blood pressure remained low for many minutes and recovery, if it occurred at all, was generally

only partial. After the injection of a still larger amount of venom (25 to 50 stings) the fall was preceded by a sharp but transient rise of pressure as shown in Fig. 7. In this experiment the blood pressure fell from about 100 mm. Hg to about 40 mm. within three minutes and rose again gradually to its original level in about an hour (Fig. 7c). This high level was not maintained and the blood pressure fell to about 70 mm. Hg. during the following hour (at e). Primary and secondary

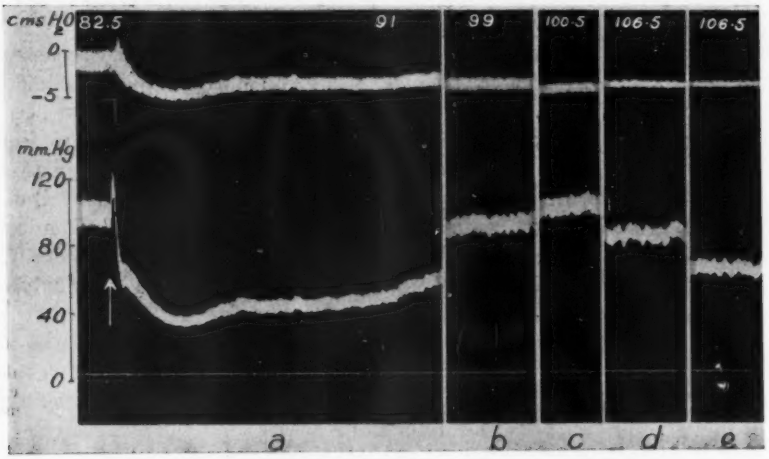


Figure 7. Venous pressure (upper tracing) and carotid blood pressure (lower tracing) from a dog weighing 10·9 kg. under chloralose. Artificial respiration. At the arrow (↑) 50 stings of bee venom (1 c.c.) intravenously. b, c, d, e in 40, 60, 90 and 120 minutes after the injection. The figures on top of the tracing represent the haemoglobin percentages. Time in half minutes.

shock can therefore be distinguished after the injection of bee venom. A similar phenomenon has been described in dogs after histamine (Smith, 1928). In some experiments, the intermediate rise of arterial pressure, which divided the two stages of shock, was less pronounced (Fig. 8).

The depressor effect of the purified venom is not caused by histamine present in the venom. The concentration of histamine in this preparation was too low to be effective as was shown by control injections of histamine. On the other hand, when a small amount of the crude venom was injected the long-lasting depressor effect was preceded by an evanescent fall of arterial blood pressure (Fig. 8B), which must be attributed to histamine. When larger doses of the crude venom were injected the initial pressor effect, which was stronger with this venom than with the purified sample, overcame the depressor action of the histamine (Fig. 9); but part of the following depressor effect must be attributed to histamine.

The dog is readily desensitized against the depressor action of the venom. By

the injection, at intervals of 5 to 10 minutes, of doses of purified venom, commencing with one sting or less and increasing by doubling, we were able finally to inject fifty stings without any depressor effect. This injection produced only a pressor response which was slight and transient.

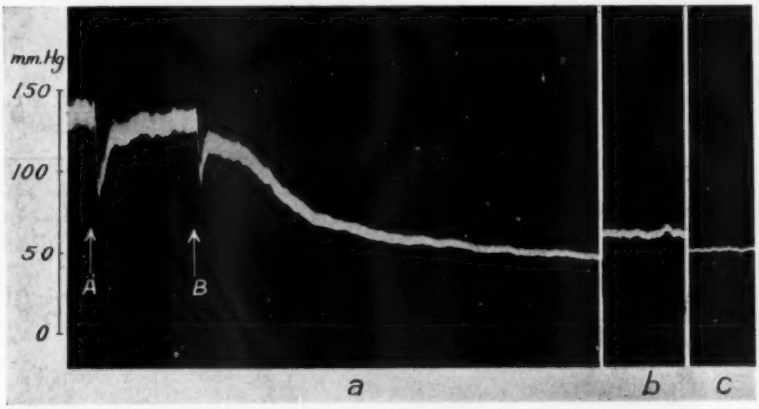


Figure 8. Carotid blood pressure tracing from a dog weighing 9·5 kg. under chloralose. Artificial respiration. Evisceration. At A, 0·1 c.c. histamine 1: 10,000, at B, 5 stings crude bee venom (0·1 c.c.) intravenously. b and c are 80 and 180 minutes after the injection of venom. Time in half minutes.

In many experiments, although the animals were adequately ventilated, natural respiratory movements occurred shortly after the injection. These were sometimes overcome by an increase in the stroke or frequency of artificial ventilation. The effects of these spontaneous respiratory movements were sometimes the only changes produced on the blood pressure tracing by a second dose of venom. These movements may have resulted from stimulation of the respiratory centre, of the chemo-receptors in the *sinus caroticus*, or of peripheral sensory nerve endings. The injection of venom was also often followed by the appearance of Traube-Hering waves.

The depressor action of the venom in the dog was not the result of heart failure nor of obstruction in the pulmonary circulation but was caused by peripheral vasodilatation associated with loss of fluid from the circulation, and was probably somewhat intensified by the effects of the venom on the liver. These conclusions are derived from the following experiments.

Venous Blood Pressure.

The fall in arterial blood pressure following the injection of bee venom is accompanied by a fall in venous pressure (Fig. 7) indicating that failure of the

heart is not responsible for the depressor action of the venom. About half-an-hour after the injection of large doses of venom (250 stings) irregularities occur in the tracing of the carotid pressure suggestive of extra systoles and of short periods of block; death may eventually occur from heart failure. Such an event seems to have taken place in the experiment of Fig. 9, in which the dog died about an hour after the injection. *Post mortem* there were subendocardial haemorrhages in the left, but not in the right ventricle. These were absent after the injection of smaller doses of venom (25 to 50 stings). The late cardiac changes observed on the arterial blood pressure tracing, after large doses of bee venom, were less striking

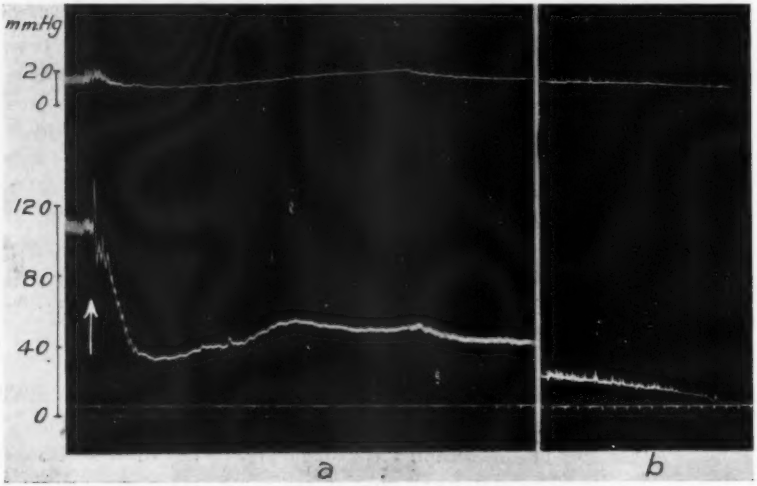


Figure 9. Blood pressure in carotid (lower tracing) and pulmonary artery (upper tracing) from a dog weighing 15 kg. under chloralose. At the arow (↑) 250 stings of crude venom (2·5 c.c.) intravenously. b, is 60 minutes after the injection. Time in half minutes.

than those described after large doses of cobra venom but this difference might, at least in part, have resulted from the fact that the arterial blood pressure often remained low for a longer period after the injection of bee venom.

Pulmonary Circulation and Lungs.

Bee venom does not cause obstruction in the pulmonary circulation of the dog. Even after large doses of the crude venom, which in cats produce strong constriction of the pulmonary vessels, the pressure in the pulmonary artery of the dog remains almost unchanged and no oedema of the lungs occurs. The lungs are somewhat congested and show areas of haemorrhage particularly in their dependent parts. In the experiment of Fig. 9, 250 stings of crude venom were injected

intravenously. The fall in arterial blood pressure was unattended by any significant change of pressure in the pulmonary artery.

Liver, Portal Circulation and Lymph Flow.

The effects of bee venom on the dog's liver resemble those of the venom of the Indian cobra. The liver is engorged as in anaphylactic and peptone shock. The changes in pressure in the portal vein and in the lymph flow are in part caused by this action of the venom.

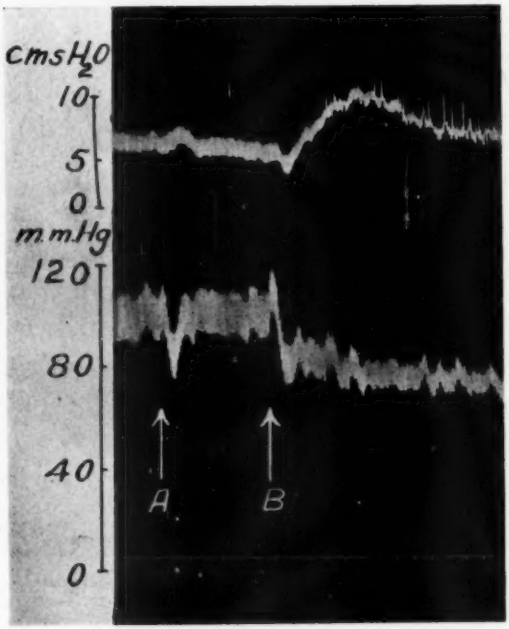


Figure 10. Blood pressure in carotid artery (lower tracing) and portal vein (upper tracing) from a dog weighing 10·9 kg. under chloralose. Artificial ventilation. At A, 0·2 c.c. histamine 1: 200,000, at B, 10 stings (0·2 c.c.) of purified bee venom intravenously. Time in half minutes.

Pressure in the Portal Vein. After the intravenous injection of a small amount of purified venom (5 stings) a rise of pressure occurs in the portal vein; after larger amounts the rise is more pronounced and may be preceded by a slight initial fall of pressure. This is illustrated in Fig. 10B. The initial fall is probably caused by vasoconstriction in the abdominal viscera (*vide infra*) and is therefore absent when the venom is injected intraportally. The rise of pressure is caused by obstruction of the liver vessels, probably of the hepatic veins, and is therefore

more pronounced when the venom is injected intraportally. Fig. 11 shows the effect of an intraportal injection of 5 stings of purified venom. The changes in the portal pressure observed in Figs. 10 and 11 were not caused by histamine present in the venom. The intravenous as well as the intraportal injection of corresponding amounts of histamine did not affect the pressure in the portal vein. It will be seen in Fig. 10A that the fall of arterial blood pressure produced by 0·2 c.c. of histamine 1 in 200,000, an amount 7½ times greater than that injected in the venom

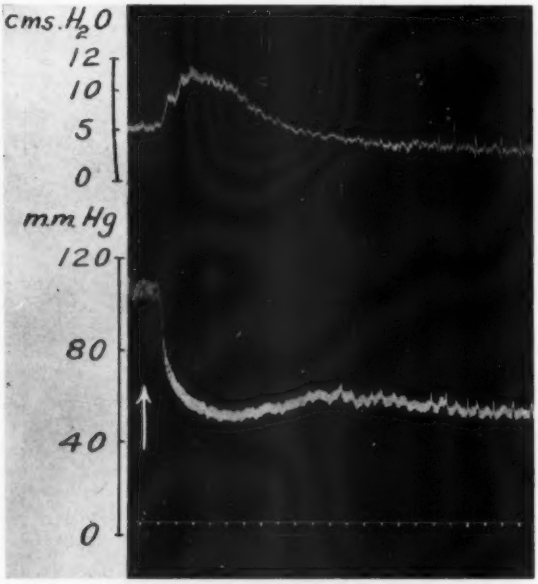


Figure 11. Blood pressure in carotid artery (lower tracing) and portal vein (upper tracing) from a dog weighing 8·2 kg. under chloralose. Artificial respiration. At the arrow (↑) 5 stings of purified venom (0·1 c.c.) intraportally.

solutions at B, was not accompanied by any significant change of pressure in the portal vein.

The liver vessels easily become desensitized to purified venom. A second intraportal injection of 10 stings produced no rise of pressure in the portal vein, and at this stage the intraportal injection of a large dose (40 stings) was ineffective.

The long-lasting depressor action on the arterial blood pressure and the ease with which desensitization is attained by successive injections of venom, render it fruitless to compare the effects of an intravenous with a subsequent intraportal injection, and to try by this means to obtain information concerning the rôle of the liver in the depressor action of the venom. The fact that the venom causes

vasodilatation, when injected arterially and produces a strong depressor action when the liver is excluded from circulation, shows that the effect on the liver can only contribute to, but is not essential for the depressor action. The depressor action, after exclusion of the liver, is illustrated by the experiment of Fig. 8. The stomach, spleen, large and small intestine had been removed and the hepatic artery tied. At B, 5 stings of crude venom were injected intravenously causing an evanescent fall of arterial pressure followed by a secondary, long-lasting and profound depressor effect from which no appreciable recovery occurred. The immediate evanescent fall of pressure is accounted for by the histamine present in the venom, since a control injection (at A) of a larger amount of histamine than that present in the venom caused a similar evanescent but somewhat greater fall of arterial blood pressure.

Lymph Flow. We have examined the effects of intraportal injections of 25 stings of purified venom. After a latent period of 1 to 3 minutes, the lymph flow increased, within 10 to 15 minutes it had become two or three times as great as before the injection; it then decreased again. Changes in the fat content of the lymph similar to those obtained after cobra venom have been observed in the samples collected during the first twenty minutes after the injection of bee venom. Twenty-five to fifty minutes after the injection, blood corpuscles and pigment from haemolysed red blood cells appeared in the lymph. The blood content of the lymph became progressively greater in the following hours, and in one experiment a haemoglobin value of 6·9 p.c., compared with the average value of 74 p.c. for dog's blood, was estimated in a sample of lymph collected five hours after the injection of the venom.

Peripheral Vessels.

These experiments, in which the changes in the venous outflow of the hind limb and bowel were studied, were carried out with purified venom. Histamine injected in amounts equivalent to those present in the venom were without effect.

Hind Limb. The injection of one sting of venom in a volume of 0·2 c.c. into the femoral artery produced an increased venous outflow which started about twenty seconds after the injection. The flow increased about twofold and the dilatation in the limb vessels persisted for from five to ten minutes. After repeated injections the vessels gradually became insensitive to this dilator action, the latent period increased and the degree and duration of the dilatation progressively diminished. Larger doses administered at this stage caused vasodilatation which was sometimes preceded by transient vasoconstriction. The constrictor effect of large doses of purified bee venom was much less pronounced than that caused by the venom of the Indian cobra.

Small Intestine. In two experiments we have studied the outflow from a loop of ileum following the arterial injection of bee venom. In one, the initial injection

of one sting caused diminution in outflow followed by dilatation, after a second larger dose (10 stings) the constriction as well as the after-dilatation were increased. In the second experiment the repeated injection of one sting caused increased outflow; the latent period increased progressively and the degree of dilatation diminished. A preceding constrictor effect was only observed after larger doses (5 stings) and was slight. In this experiment the arterial injection of 1 mg. of cobra venom stopped the outflow for some minutes. These results are in accord with the inconstancy of the fall of portal pressure observed after intravenous injections of bee venom, and afford further proof that purified bee venom has a less powerful constrictor action than has the venom of the Indian cobra.

In addition to these changes in the venous outflow, we have constantly observed, after intravenous and intraportal injection of large doses of venom (25 to 100 stings), changes in the mucosa of the small intestine identical with those we have described for cobra venom, peptone and histamine (1937d). These included an intense haemorrhagic congestion, and microscopic examination showed the capillaries in the villi to be distended with blood. The changes were most pronounced in the duodenum and upper portion of the jejunum, and diminished in intensity in the ileum. The lower part of the ileum and the large bowel were only congested. The normal or slightly congested mucous membrane of the stomach was clearly marked off from the intensely congested and haemorrhagic duodenal mucosa. The lesions are illustrated in Fig. 12, which are from photographs of colour preparations of the pylorus and first part of the duodenum from dogs killed 170 minutes after the intravenous injection of five stings of bee venom per kg. (a), 75 minutes after 0·4 mg. of histamine per kg. (b), and 35 minutes after 0·5 mg. of cobra venom per kg. (c). All showed haemorrhagic changes of the same kind in the duodenal mucosa in contrast to the normal appearance of the mucosa of the stomach. The difference in intensity of the changes may be related to differences in dosage and time of action. We have observed, after cobra venom and histamine, changes as striking as those figured from bee venom; and after bee venom, changes of the same intensity as those reproduced in (B) and (C). In those experiments in which the haemorrhages in the duodeno-jejunal mucosa were less massive, the lesions tended to be arranged in narrow bands parallel to the long axis of the gut. There was fresh blood in the lumen of the small and of the large bowel, and faeces mixed with blood were passed in many experiments.

In one experiment, we desensitized a dog by a number of small but increasing doses of venom (1 to 50 stings). When the animal was killed, half-an-hour after the last injection, the changes in the gut were relatively slight, being confined to the duodenum, where a few small haemorrhages were found. Desensitization against the damage produced by the venom in the vessels of the mucosa of the small intestine is therefore possible. We have recently obtained a similar result with cobra venom.

Figure 12. Photographs of the pylorus and first part of the duodenum in dogs killed (a) after bee venom, (b) after histamine and (c) after cobra venom had been injected intravenously. (For details see text.)

Fluid Loss from Circulation.

Bee venom causes loss of fluid from circulation as a result of changes in the permeability of the capillaries. This is shown by the increase in haemoglobin concentration of the blood. In the experiment of Fig. 7, the haemoglobin percentage rose from 82·5 before the injection to 106·5 (i.e. an increase of 29 p.c.) one-and-a-half hours after the injection. There was no further change during the following hour. The abdomen was then opened and the spleen was found to be distended. Contraction of this organ cannot therefore have contributed to the observed concentration. In another experiment, in which the spleen was removed before the injection of purified venom (50 stings), the haemoglobin percentage rose from 67 p.c. to 82·5 (i.e. 23 p.c.).

Liberation of Histamine from Perfused Liver and Lungs.

Since in dogs the liver can be regarded as a "shock organ" for many cell-injurious agents and was found to show profound changes after the intravenous injection of bee venom, we used this organ first for studying the liberation of histamine. On the other hand the changes in the dog's lung, after the intravenous injection of bee venom, were found to be trivial, indicating that it is rather insensitive to the cell-injurious action of the venom and therefore particularly suitable for a control experiment. However, contrary to our expectation, the lung not only yielded a high histamine equivalent on extraction, but this histamine was also set free from it during perfusion after the injection of venom.

Histamine Equivalent of Liver. Saline extracts made from a few grams of liver were assayed for histamine on the isolated gut of the guinea-pig. Since the liver is known to contain acetylcholine and choline, the possibility that part of the gut-contracting action of the extracts resulted from the presence of these substances had to be taken into account. Acetylcholine was excluded by treating the extract with alkali, which did not diminish its activity. That is not surprising since the extraction is made under conditions which favour the hydrolysis of acetylcholine. The presence of choline in effective concentrations was rendered improbable by the fact that the amounts of extract necessary for the assay corresponded to between 0·25 and 1 mg. of tissue only. Choline could be excluded when it was found that the extract had a strong depressor action in the cat, which was not diminished by atropine. The identification of the active substance as histamine rested upon the observation that the gut-contracting and depressor actions of a given extract matched with the same histamine concentration, and upon the fact that histamine has been isolated from dog's liver and identified chemically (Hanke and Koessler, 1924; Lucas, 1926; Best, Dale, Dudley and Thorpe, 1927) in amounts of the same order as those which we have determined by biological assay. We shall therefore refer to the active principle as histamine.

In one experiment we made extracts from four different parts of the liver

using for each about 2 gm. of tissue. The histamine values obtained per gram of tissue did not differ by more than 20 p.c. The value obtained from an extract made from a small piece of liver may thus be taken as indicating the histamine concentration in the whole organ. We generally used between 2 and 5 gm. for extraction and found that the histamine content per gram of liver varied in different animals between 20 and 110γ.

Liver Perfusion. When a piece of liver was perfused without the addition of venom, the venous perfusate, if added in amounts of 0·2–0·4 c.c. to the 3 c.c. bath, had usually no action on the isolated gut. In some experiments the perfusate

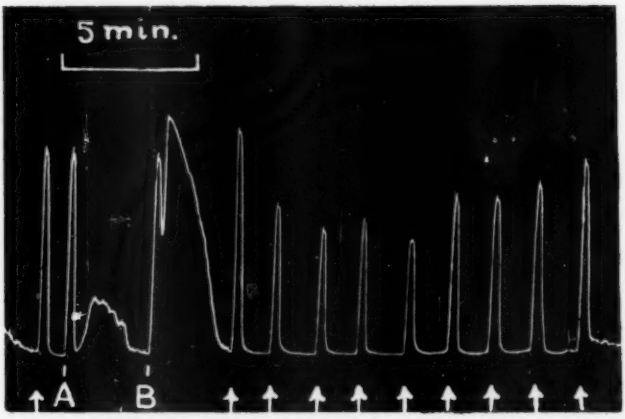


Figure 13. Isolated guinea-pig jejunum in 3 c.c. bath. At the arrows (↑) 0·06γ of histamine. At A and B, 0·1 c.c. of perfusate collected in the fifth hour after the injection of bee venom. Same experiment as in fig. 14.

caused a delayed slow contraction, which sometimes did not start until the fluid in the bath had been changed. In one experiment only the perfusate caused a quick, immediate contraction corresponding to a histamine content of about 1 in 15 millions. This result was obtained from a liver yielding a high histamine equivalent (110γ per gram) on extraction. In those experiments in which the perfusate was devoid of activity it remained so during prolonged perfusion. In one experiment, for instance, a piece of liver, containing 90γ of histamine per gram, was perfused for nine hours without the injection of venom and the perfusate collected in hourly samples remained throughout devoid of any activity on the gut. The outflowing fluid in the early stages of perfusion contained some coagulable protein and blood, but as perfusion proceeded the perfusate became free from blood and the amount of protein diminished, though the fluid was still somewhat turbid after many hours of perfusion. Prolonged perfusion did not lead

to accumulation of any perceptible quantity of fluid in the tissue and the concentration of histamine in the liver was unaltered.

The injection of venom (5 to 20 stings) into the portal vein caused the appearance in the perfusate of a substance causing rapid and immediate contraction of

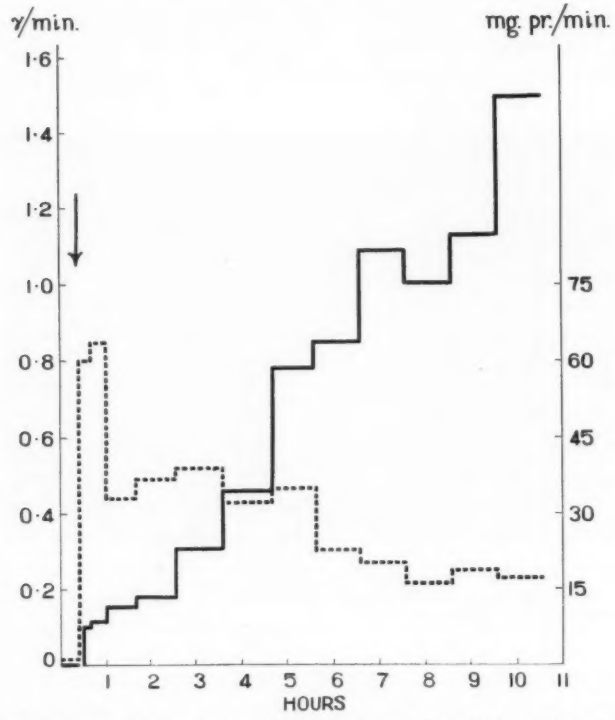


Figure 14. Output of histamine (continuous line) and of protein (dotted line) from 36 gm. of perfused dog's liver. Abscissae, time in hours. Ordinates on the left output of histamine in γ per minute, on the right output of protein in mg. per minute. At the arrow (↓) injection of 5 stings of purified bee venom.

the gut, of a substance which caused a slow and delayed contraction, of coagulable protein and of yellowish-brown pigment.

The substance which caused rapid contraction was identified pharmacologically as histamine in the same manner as in the experiments on perfused guinea-pig's lungs. We were further able to show that it was part of the liver histamine.

The substance, which caused a slow and delayed contraction of the gut, was not identified. Contrary to our findings in experiments on the guinea-pig's lung

and, as we shall see later, in those on the dog's lung, it was greatly reduced in activity and sometimes completely destroyed by boiling. This is illustrated in Fig. 13 which shows the effect on the gut of 0·2 c.c. of active perfusate before (B) and after (A) boiling. The figure also illustrates the changes in reactivity of the gut to histamine which often followed the addition of active perfusate. During the first one or two minutes after the slow contraction had subsided the sensitivity of the gut to histamine was increased, later it decreased. Sometimes increased and sometimes decreased sensitivity was more pronounced. These changes had to be taken into account in the assay of the perfusate.

Histamine appeared in the perfusate within the first few minutes after the injection of venom and continued to be present in increasing amounts for many hours. This is illustrated in Figs. 14 and 15. In the experiment of Fig. 14, 36 gm. of liver from an 8·4 kg. dog were perfused for ten hours after the injection of five stings of venom. The liver contained per gm. 32γ of histamine. Before the injection of venom the perfusate was devoid of activity on the gut, but after the injection histamine appeared in the perfusate and the output increased from 0·11γ per minute in the first half hour to 1·52γ per minute in the tenth hour. The total amount of histamine estimated in the perfusate amounted to 454γ. The liver was now removed and allowed to drain for half an hour, an additional 15γ being assayed in the drainage fluid. After drainage for half an hour there was no perceptible accumulation of fluid in the liver which weighed approximately the same as before the perfusion. The histamine content of the liver had fallen from 32γ to 19γ per gram, corresponding to a histamine loss of 468γ. This is an unusually good correspondence, but within a range of usually less than 20 p.c. the total amount of histamine estimated in the perfusates and drainage fluid corresponded to the loss calculated by the difference between the histamine content of the liver before and after perfusion. This shows that the histamine assayed in the perfusate and drainage fluids is not newly-formed in the liver by the venom but is liberated from the tissue and represents part of the original store of histamine in the liver. The histamine output of 468γ in this experiment amounted to 40 p.c. of the liver histamine and was still increasing at the end of the perfusion. We have therefore, in one experiment, continued perfusion for 17½ hours after the injection of venom (Fig. 15). It will be seen that the output of histamine increased during the first eight hours, remained at a high level (about 1·5γ per minute) for the succeeding six hours and then decreased somewhat. Even after 17½ hours the output amounted to about 1γ per minute. The decrease is less striking if the output is expressed as percentages of the histamine available for liberation in the liver at the times when the different samples were taken. For instance, at the eleventh hour 0·122 p.c. of the amount of histamine remaining in the liver was found per minute in the perfusate, this value decreased to 0·093 p.c. at the end of the perfusion. In this experiment 1,119γ of histamine were recovered in the perfusate collected from 76·6 gm. of liver, the histamine content of which had fallen from 32γ to 12·5γ

per gram, a loss of 1,494γ. The difference between the calculated histamine loss from the liver and that recovered in the perfusate and drainage fluid amounted to 25 p.c. an unusually high discrepancy. In this experiment it will be noted that the histamine output per minute of the second sample is higher than that of the following four samples. Such an initial peak in the output has been observed in most experiments. If the drainage fluid is collected in successive samples, the concentration of histamine in them increases and there is a gradient from the concentrations in the last sample of perfusate to that in the liver indicating increased

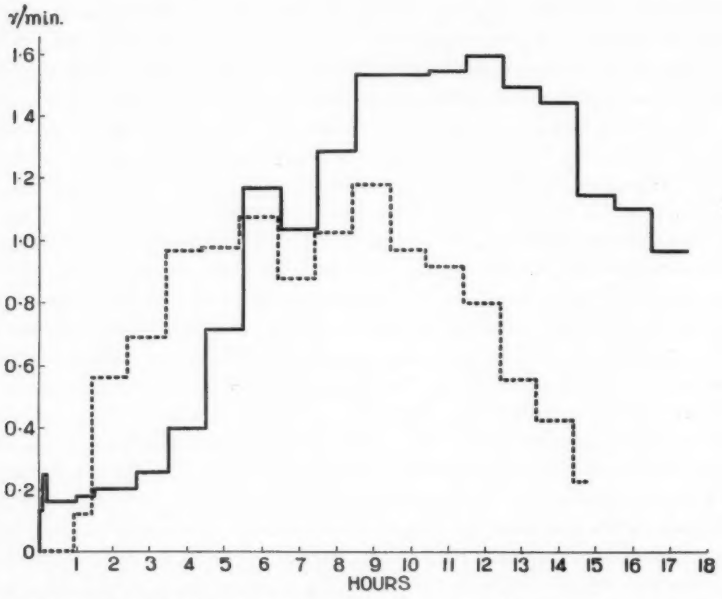


Figure 15. Output of histamine from 76·6 gm. of perfused liver (continuous line) and 22 gm. of perfused lung (dotted line) from a dog after the injection of 10 and 15 stings of bee venom respectively. Abscissae time in hours after the injection of venom. Ordinates output of histamine in γ per minute.

permeability of the cells to their histamine. In the experiment of Fig. 14, for instance, the last sample of perfusate had a histamine concentration of 1 in 1·4 millions. During the first ten minutes of drainage 6·4 c.c. of fluid containing histamine in a concentration of 1 in 600,000, and, during the ensuing twenty minutes, 1·3 c.c. of fluid with a concentration of 1 in 300,000 were collected. The concentration of histamine calculated for the liver was 1 in 53,000. Further indication of the increase in permeability of the liver cells to their histamine is afforded by the ratio of the histamine concentration of the last sample of drainage fluid to that of the liver. In the experiment just described the histamine concen-

tration in the last sample of drainage fluid was about six times weaker than that calculated for the liver. In the experiment of Fig. 15 this ratio was 1 : 5. In control experiments in which no venom was administered the ratio was about 1 : 100. For example, in the experiment mentioned above in which a piece of liver, containing 90γ of histamine per gram was perfused for nine hours, the drainage fluid collected at the end of the perfusion, amounted to 3·7 c.c. in the first ten minutes and to 1 c.c. in the following twenty minutes. The concentrations of histamine in these two samples were 1 in 1·7 million and 1·1 million. The histamine concentration calculated for the liver was 1 in 11,000.

The coagulable protein in the perfusate was roughly estimated with Esbach's reagent. In Fig. 14 the output is given in mg. per minute. It will be seen that the output is large in the first half-hour after the injection, falls off rapidly in the immediately succeeding period and then more gradually till the end of the experiment. This was a regular finding. Contrary to the results obtained with the guinea-pig's lung the maximum liberation of protein precedes that of histamine.

After the injection of bee venom the perfusate became yellowish-brown in colour from the appearance of pigments in the perfusate. The brownish tint decreased ten to fifteen minutes after the injection and disappeared completely in the course of the next fifteen to sixty minutes. The concentration of pigments in the perfusate reached its highest level before the output of coagulable protein had attained its maximum. This phenomenon may be related to the late appearance of bile pigment in the lymph collected from the thoracic duct of dogs after the intravenous injection of histamine (Feldberg and Kellaway, 1937d).

Histamine Equivalent of Lung. The histamine content of the dog's lung, estimated pharmacologically, varied between 20γ and 220γ per gram of tissue. In most experiments it was higher than in the liver of the same animal. The assay of histamine on the isolated small intestine of the guinea-pig was more difficult than in the case of liver extracts or of extracts of guinea-pig's lung, because the substance causing slow and delayed contraction was present in apparently greater amount, could not be destroyed by boiling, and caused the characteristic alterations in the sensitivity of the gut.

Lung Perfusion. A lung can be perfused for many hours without gross change in its appearance. There are no glassy areas of atelectasis and no great accumulation of fluid in the lung. The perfusate remains free from detectable amounts of histamine, but sometimes the samples collected early in the perfusion, if added in a dose of 0·4 c.c. to the bath containing the guinea-pig's intestine, cause delayed and strong contractions from which the gut recovers only slowly after changing the Tyrode solution. In one experiment for instance we perfused a lung weighing 44 gm. for 6½ hours. The lung contained 30γ of histamine per gram. No histamine appeared in the perfusate and no loss had occurred during the perfusion. When the lung was removed it was allowed to drain for 30 minutes; 3·2 c.c. were collected in the first fifteen minutes but there was no further fluid drain-

age. The lung weighed nearly 9 gm. more than before the perfusion; a total of 12 c.c. of fluid had therefore accumulated. The histamine concentration in the drainage fluid was 1 in 2·5 millions and that calculated for the lung from its histamine content (25γ per gram) at the end of the experiment was 1 in 40,000.

The injection of bee venom, 10–20 stings, into the pulmonary artery of the perfused lung caused the appearance of histamine in the perfusate after a latent period which varied from ten minutes to one and a half hours. This histamine was identified pharmacologically in the same manner as described for the guinea-pig's lung, and could be shown to be part of the lung histamine. The assay of the perfusate on the gut was made difficult by the presence of the substance causing slow contraction which was not destroyed or even appreciably diminished in activity by boiling. The histamine output from one experiment is illustrated in Fig. 15, in which 22 gm. of lung (two lobes) containing 40γ of histamine per gm. were perfused for nearly fifteen hours after the injection of 15 stings of venom in 0·3 c.c. The perfusate collected during one hour before the injection contained no demonstrable amount of histamine. It will be seen that no histamine appeared in the samples of perfusate collected during the first 50 minutes after the injection of venom. In the following samples the concentration of histamine increased and the output reached about 1γ per minute in the fourth hour, remained at this high level for about six hours and then decreased again. Twenty minutes after the injection the lung began to swell and glassy patches appeared on its surface. The outflow was less than the inflow, and by the end of the experiment enough fluid had accumulated in the lung to increase its weight sevenfold. A large part of this fluid drained out in the course of a few minutes, when the bronchi were opened at the end of the perfusion.

The total output of histamine in this experiment was 669γ and an additional 22γ were recovered in the drainage fluid collected during 1 hour and 40 minutes. The lung after drainage weighed 43 gm. so that it still contained 21 c.c. of fluid. The histamine remaining in this lung was 301γ and the amount lost should have been 579γ instead of 691γ actually estimated in the perfusates and drainage fluid. This difference is about 20 p.c. and lies within the errors of the assays. The histamine in the perfusate is therefore derived from the lung.

The drainage fluid in this experiment was collected in four successive samples in 5, 10, 35 and 50 minutes, measuring 59, 26, 12 and 4·5 c.c. respectively. The histamine concentrations of these samples were 1 in 10 millions, 1 in 5 millions, 1 in 2 millions and 1 in 900,000, and that calculated for the lung itself was 1 in 140,000. The ratio between the histamine content of the last sample of drainage fluid and that in the lung was 1 : 6·4 compared to a ratio of 1 : 60 in the lung perfused for six and a half hours without the injection of venom.

After the injection of venom a small amount of coagulable protein appeared in the perfusate; it reached its maximum during the first two hours and the fluid became free from measurable amounts before the histamine output had reached its summit.

DISCUSSION.

Analysis of the pharmacological actions of bee venom on the circulation and on isolated smooth muscle has shown that the effects which result from the presence of histamine in the venom must be differentiated from those which are brought about by its other constituents. The symptoms resulting from these latter are to some extent the outcome of cell injury which leads to the liberation of histamine from the tissues. Histamine therefore plays a double rôle in the symptomatology of bee venom poisoning.

In different species there is evidently great variation in the sensitiveness of the cells to injury brought about by the venom. In the cat the cells are highly resistant to this kind of injury. The large amount of histamine present in crude venom contributes greatly to the circulatory effects in this species and, if the venom is injected in small quantities, is solely responsible for the symptoms. In the dog, on the other hand, the cells are sensitive to injury by the venom and long lasting circulatory changes are produced by small doses. The histamine present in these, even if crude venom be used, causes only immediate evanescent effects.

The assumption that the long-lasting circulatory effects can in part be attributed to the liberation of histamine rests, not only upon the similarity of the symptoms to those produced in histamine poisoning, but also upon the actual demonstration of its liberation from perfused organs. The liberation of histamine from the lung of the guinea-pig and from the liver of the dog fits well with the site of production of symptoms in these species. The long-lasting output of histamine from the perfused liver of the dog after the injection of venom and the long continued and increasing escape of blood into the lymph collected from the thoracic duct of the intact animal are in accord with the production and persistence of severe injury to the liver. On the other hand the liberation of large amounts of histamine from the perfused lung of the dog and the relative insensitiveness to venom of this organ and its vessels *in situ*, present a difficulty for which at present we can only offer a suggestion. It is conceivable that the cells of the artificially perfused lung are less resistant to injury by the venom and more sensitive to the action of histamine.

In comparing the symptoms produced by histamine and by bee venom the direct effect of cell injury and the local action of the liberated histamine have to be taken into account. These considerations have already been discussed in our previous communications on snake venoms (1937 b, c, and d).

Bee venom resembles cobra venom very closely in its effects upon the circulation and upon the lungs. In the cat this resemblance is particularly striking if crude venom is injected in large amounts. The effects on the blood pressures in the carotid and pulmonary arteries are indistinguishable from those produced by a lethal dose of cobra venom injected intravenously. A comparison of Fig. 4a of this paper with Fig. 1 on page 161 of this volume illustrates this point. Furthermore the gross changes produced in the lungs by cobra venom and by bee venom,

either crude or purified, are closely similar. In the dog the parallelism in the actions of these two venoms is equally striking. Both cause peripheral vasodilatation and loss of fluid from the circulation. Both produce similar changes in the liver and lymph flow, haemorrhagic congestion of the duodenum and subendocardial haemorrhages. The lung vessels are relatively insensitive to both poisons. There are, however, some differences in the circulatory effects of the venoms. In the dog the depressor action of bee venom is more prolonged than that of cobra venom and the changes in the activity of the heart are less pronounced. This may result from a difference in the nature of the local cell injury caused by the venom or from the presence of a stronger constrictor element in cobra venom. In different samples of bee venom the vaso-constrictor action varies according to the kind of preparation. It is reduced in the purified samples, which have a much weaker constrictor effect than cobra venom. To estimate the constrictor activity in crude venom is difficult because it is masked by vasodilatation caused by the large amounts of histamine present.

SUMMARY.

1. In analysing the effects of bee venom we have to differentiate between the actions of histamine present in the venom and those of other constituents.

2. The histamine present in bee venom causes a rapid contraction of the isolated jejunum of the guinea-pig. This is followed by a slow and prolonged contraction and after recovery by characteristic alterations in reactivity to histamine.

3. In the cat large doses of purified venom are necessary to produce profound and long-lasting changes. Such doses, injected intravenously, cause a fall in systemic blood pressure, a rise of pressure in the pulmonary artery, fluid loss from circulation and sometimes heart failure. The lungs may be intensely congested and haemorrhagic. When crude venom containing much histamine is used, the depressor effect of small doses is wholly accounted for by histamine.

4. In the dog the intravenous injection of small doses of venom causes a long-lasting fall of systemic blood pressure with fluid loss from circulation. If crude venom is used this effect is preceded by a transitory fall of pressure resulting from the presence of histamine in the venom. The pressure in the pulmonary artery is not altered but that in the portal vein is increased. The liver is engorged. the lymph flow increased and the lymph collected from the thoracic duct contains blood in increasing amounts during the course of several hours. *Post mortem*, there are subendocardial haemorrhages in the left ventricle; the mucosa of the duodenum shows haemorrhagic congestion and there is free blood in the lumen of the bowel.

5. Bee venom causes a long-lasting liberation of histamine from the perfused lungs of the guinea-pig and dog and from the perfused liver of the dog. The

output of histamine from the guinea-pig's lung reaches its maximum in the first ninety minutes and that from the dog's organs, eight to ten hours after the injection of venom. From between 40 to 75 p.c. of the histamine in the organs is liberated by venom in a dose of 5 to 20 stings. Coagulable protein appears in the perfusate and when the liver is perfused pigments are also liberated.

REFERENCES.

Beck, B. F. (1935): Bee Venom Therapy, New York.
Best, C. H., Dale, H. H. and Thorpe, W. V. (1927): J. Physiol., 62, p. 397.
Essex, H. E., Markowitz, J. and Mann, F. C. (1930): Amer. J Physiol., 94, p. 209.
Feldberg, W. and Kellaway, C. H. (1937a): J. Physiol., 90, p. 257.
Feldberg, W. and Kellaway, C. H. (1937b): Austral. J. exp. Biol., 15, p. 81.
Feldberg, W. and Kellaway, C. H. (1937c): Austral. J. exp. Biol., 15, p. 159.
Feldberg, W. and Kellaway, C. H. (1937d): Austral. J. exp. Biol., 15, p. 441.
Feldberg, W. and Keogh, E. V. (1937): J. Physiol., 90, p. 280.
Hanke, M. T. and Koessler, K. K. (1924): J. biol. chem., 59, p. 879.
Kellaway, C. H. and LeMessurier, D. H. (1936): Austral. J. exp. Biol., 14, p 57.
Langer, J. (1897): Arch. exp. Path. Pharmak., 38, p. 381.
Lucas, G. H. W. (1926): Amer. J. Physiol., 77, p. 114.
Morgenroth, J. and Carpi, U. (1906): Berl. klin. Wschr., 43, p. 1424.
Nagamitu, G. (1935): Okayama Igakkai Zasshi, 47, p. 3005, quoted after Ber. ges. Physiol. (1936) 93, p. 548.
Phisalix, N. (1922): Animaux venimeux et venins, Paris, Masson, p. 387.
Reinert, M. (1936): Festschrift f. E. Barell, Basel, p. 407.
Smith, M. J. (1928): J. Pharmacol., 32, p. 465.
Tetsch, C. and Wolff, K. (1936): Biochem. Z., 288, p. 126.

End.